MAP Kinase Signaling Protocols

Edited by

Rony Seger

1

The ERK Cascade As a Prototype of MAPK Signaling Pathways

Hadara Rubinfeld and Rony Seger

1. Introduction

Sequential activation of kinases (protein kinase cascades) is a common mechanism of signal transduction in many cellular processes *(1)*. Over the past decade several related intracellular signaling cascades have been elucidated, collectively known as mitogen-activated protein kinase (MAPK) signaling cascades *(2–7)*. These cascades cooperate in transmitting extracellular signals to their intracellular targets and thus initiate cellular processes such as proliferation, differentiation, development, stress response, and apoptosis. Each of these signaling cascades consists of three to six tiers of protein kinases that sequentially activate each other by phosphorylation. The similarity between the enzymes that comprise each tier in the various cascades makes them a part of a superfamily of protein kinases.

The MAPK cascades are activated either by a small guanosine 5'-triphosphate (GTP)-binding protein (smGP; Ras family protein) or by an adapter protein, which transmits the signal either directly or through a mediator kinase (MAP4K) to the MAPK kinase kinase (MAP3K) level of the cascades (**Fig. 1**). Subsequently, the signal is transmitted down the cascade by enzymes located at the following tiers, which are referred to as MAPK kinase (MAPKK), MAPK, and MAPK-activated protein kinase (MAPKAPK). The four to five tiers in each of the MAPK cascades are probably essential for signal amplification, specificity determination, and tight regulation of the transmitted signal. More important, all the enzymes at any given level share common phosphorylation sites, which often lie within an area called the activation loop or activation lip *(8)*. In the case of the MAPK level, the phosphorylation sites are threonine (Thr) and tyrosine (Tyr), arranged in a Thr-Xaa-Tyr motif *(9)*, that is usually used to distinguish the individual cascades.
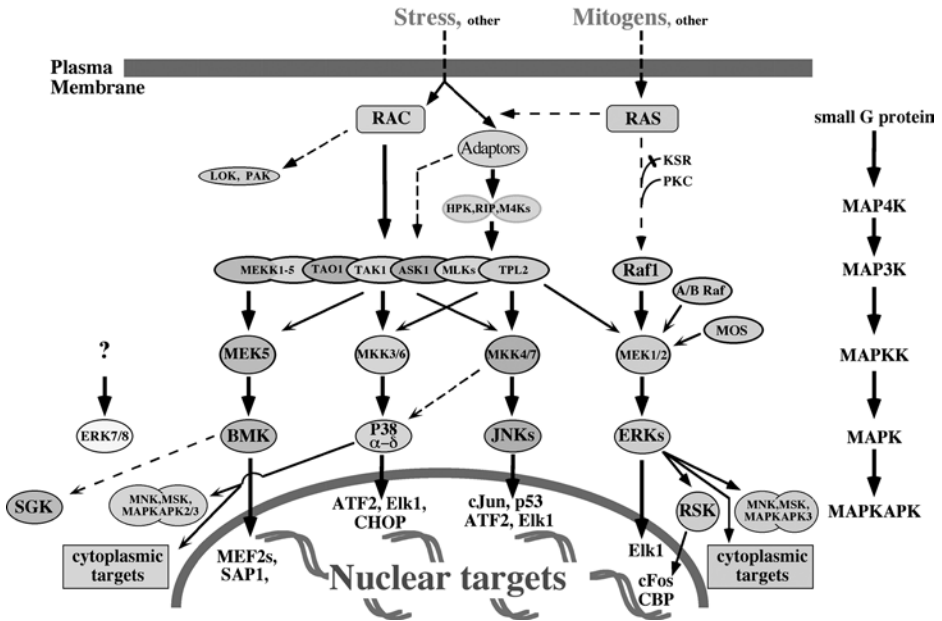
Fig. 1. Schematic representation of MAPK signaling pathways.

The four distinct MAPK cascades currently known are named according to the subgroup of their MAPK components: (1) extracellular signal-regulated kinase (ERK) *(10)*; (2) c-Jun N-terminal kinase (JNK), also known as stress-activated protein kinase 1 (SAPK1) *(11,12)*; (3) p38MAPK, also known as SAPK2–4 or p38α–δ *(13–15)*; and (4) Big MAPK (BMK), also known as ERK5 *(16,17)*. In each of the cascades, the MAPK level is composed of several very similar isoforms, which may provide a broader range of activity to the cascades. The different groups of MAPKs seem to differ in their physiologic activities. Usually, the ERKs play a role in proliferation and differentiation, whereas the other cascades seem to respond to stress and are involved in apoptosis. However, some of the functions of each of the cascades are cell type and cell condition specific, and it has been shown that ERKs, which are usually involved in cellular proliferation, may participate in certain cell types in the response to stress and apoptosis *(18)*.

1.1. ERK Cascade

ERKs are activated by a variety of extracellular agents, which include, among others, growth factors, hormones, and neurotransmitters *(4)*. The extracellular factors, which can act through heterotrimeric G-coupled receptors *(19)*, tyrosine kinase membranal receptors *(20)*, ion channels *(21)*, and more *(5)*, can

initiate a variety of intracellular signaling events that result in activation of the ERK cascade. This activation often requires adapter proteins, which are linked to guanine exchange factors (GEFs) of small GTP-binding proteins. Upon stimulation, the adapter protein–GEF complex is recruited to the plasma membrane, where it induces activation of the small GTP-binding protein itself (e.g., Ras, Rap), which further transmits the signal to the MAP3K level of the cascade (Raf1, B-Raf, and possibly also A-Raf, MEKKs, and TPL2). For example, mitogenic stimulation induces the accumulation of active GTP-bound Ras, which in turn recruits Raf-1 to the plasma membrane, where it is activated by a mechanism that is not yet fully understood *(22)*. MOS is another MAP3K of the ERK cascade, but it operates mainly in the reproductive system by a distinct mode of regulation *(23)*. Thereafter, the signal is transmitted down the cascade through several similar MAPK/ERK kinases (MEKs) (MEK1 and MEK2, and possibly also MEK1b). In this cascade of events, the MEKs are phosphorylated and activated by Raf and other MAP3Ks through serine phosphorylation at the typical Ser-Xaa-Ala-Xaa-Ser motif in their activation loop (Ser 218, 222 in MEK1; *[24]*). The activated MEKs are dual-specificity kinases, which demonstrate a unique selectivity toward ERKs in the MAPK level *(25)*. Three ERKs (ERK1, ERK2, and ERK1b) have been identified thus far as ubiquitous Ser/Thr kinases that participate in many signaling processes. The activation of the ERKs is executed by phosphorylation of both Tyr and Thr residues in the Thr-Glu-Tyr motif in the activation loop of ERKs, and this appears to occur exclusively by MEKs. At this stage, the signal is transmitted either to regulatory proteins, described below, or to one or more of the Ser/Thr kinases at the MAPKAPK level. This group of protein kinases includes the ribosomal S6 kinase (RSK) *(26)*, the MAPK/SAPK-activated kinase (MSK) *(27)*, and MAPK signal-interacting kinase 1 (MNK1) *(28,29)*, although the two latter ones can also be activated by p38MAPK. Finally, protein kinases such as GSK3 *(30)* and LKB1 *(31)* have been identified as immediate substrates for MAPKAPKs, completing a plausible six-tier MAPK kinase (PKC/Raf/MEK/ERK/RSK/GSK3).

1.2. p38MAPK Cascade

The p38MAPK cascade seems to participate primarily in the response of cells to stress. Many kinases at the MAP3K and MAP4K levels have been implicated in the p38MAPK cascade (**Fig. 1**); however, their individual roles are not yet known. Thus, 10 or more distinct kinases have been implicated at the MAP3K level of this cascade (MEKK1–5, MTK1, MLK3, TPL2, TAO1, DLK, and TAK1; reviewed in part in **ref. 32**). At the MAPKK level, MKK6 (SKK3, SKK6, MEK6), MKK3 (SKK2), and possibly also MKK4 (SKK1, SEK1, JNKK1) are responsible for activation of all p38MAPKs (reviewed in

ref. 33). They are activated by phoshorylation at the typical Ser-Xaa-Ala-Xaa-Thr motif in their activation loop (Ser207, Thr211 in MKK6). The MAPK level components of this cascade are p38MAPKα (also known as RK, Hog, SAPK2a, and CSBP), p38MAPKβ (SAPK2b), and also p38MAPKγ and δ (SAPK3 and SAPK4) *(14,15,34–36)*. p38MAPK genes probably have several alternatively spliced forms, bringing the number of isoforms of this group to nine, and all are activated by phosphorylation of the Tyr and Thr in the Thr-Gly-Tyr motif in their activation loop. Once these p38MAPKs are activated, they transmit the signal either to the MAPKAPK level components MAPKAPK 2 and 3 *(37,38)*, MNK, MSK (as for ERKs), and PRAK *(39)*, or they phosphorylate regulatory molecules such as phospholipase A_2 (PLA_2) *(40)*, and the transcription factors ATF2, ELK1, CHOP, and MEF2C *(32)*. MAPKAPKs can then either phosphorylate heat-shock and other regulatory proteins *(15)* or complete a plausible six-tiered cascade by phosphorylating protein kinases such as LKB1.

1.3. JNK (SAPK1) Cascade

Other stress-activated MAPKs include the c-Jun NH2-terminal kinases (JNKs, also termed SAPK1; *[41]*), which constitute a third MAPK subgroup. However, these enzymes are not closely related to p38MAPK, and these two cascades are not simultaneously activated upon extracellular stimulation. Like the other MAPK cascades, this cascade can be triggered by small GTPases *(42)* that lead the signals to the MAP3K level. Alternatively, some adapter proteins can activate this cascade by phosphorylating kinases at the MAP4K level (reviewed in **ref. 43**), which in turn activate several MAP3Ks that are apparently shared by the p38MAPK cascade. At the MAPKK level, two dual-specificity enzymes, MKK4 (SKK1, SEK1; *[44]*) and MKK7 (JNKK2; *[45,46]*), can lead to the activation of JNKs. These two JNKKs are activated by phosphorylation at the typical Ser-Xaa-Ala-Xaa-Thr motif in their activation loop (Ser198, Thr202 in MKK7). The JNKKs are able to activate the components at the MAPK level, JNK1–3 (SAPKs; *[12,47]*), which have molecular masses of 46, 54, and 52 kDa, respectively. The activation loop of JNKs contains a proline in the Xaa position of the Thr-Xaa-Tyr motif, and, as with the other MAPKs, both Thr and Tyr need to be phosphorylated to achieve activation. Only a small number of MAPKAPK and cytosolic targets have been identified for JNKs *(48,49)*, but these enzymes appear to be major regulators of nuclear processes, in particular transcription. Shortly after activation, JNKs translocate into the nucleus, where they physically associate with, and activate, their target transcription factors (e.g., cJun, ATF, Elk; *[41]*). Interestingly, groups of components in this cascade appear to be held together by several scaffold proteins *(41)*, which provide their specificity in various types of external stimulations.

1.4. BMK (ERK5) Cascade and ERK7

Another MAPK subgroup consists of the BMKs (BMK1, ERK5 *[16,17]*) having a molecular mass of 110 kDa. The direct upstream activator of BMK1 is EK5 *(16)*, whereas TPL2 *(50)*, MLTK *(51)*, and MEKK2/3 *(52,53)* operate at the MAP3K level, although the exact mechanism of activation at that level is not yet clear. Since MEK5 contains a Ser-Xaa-Ala-Xaa-Thr motif in its activation loop, which is characteristic of stress-activated MAPKKs, it was initially speculated that MEK5-BMK1 is activated by stress-related stimuli. Indeed, it was found that ERK5 is activated by oxidative stress and hyperosmolarity *(17)*. However, it was subsequently shown that ERK5 could be activated also by mitogens such as serum and the growth factors epidermal growth factor (EGF) and nerve growth factor (NGF) (reviewed in **ref. 54**). The activation loop of BMK1 contains the sequence Thr-Glu-Tyr, which is identical to that of ERK1 and ERK2, and both Tyr and Thr need to be phosphorylated for activation of the enzyme. However, in spite of the similarity in the activation motif, BMK1 cannot be phosphorylated or activated by MEK1 and 2. Upon serum stimulation, BMK1 phosphorylates the transcription factor MEF2C. This factor, together with the AP-1 transcription factor, can induce the transactivation of the c-Jun gene, which contains MEF2C-binding elements on its promotor *(54)*. Interestingly, it was shown that BMK1 can serve as a transcription factor, so it can regulate transcription by itself *(55)*. Other substrates of this cascade are the transcription factors Sap1, MEF2B, and MEF2D *(54)*, and it was reported that also the serum- and glucocorticoid-responsive kinase SGK *(56)* may lie downstream of this cascade.

Another member of the MAPK family has been cloned and characterized, termed ERK7 (61 kDa; *[57]*). Although it has the signature Thr-Glu-Tyr activation motif of ERK1 and ERK2, ERK7 is not activated by extracellular stimuli that typically activate ERK1 and ERK2 or by common activators of JNK and p38MAPK. Instead, ERK7 has appreciable constitutive activity in serum-starved cells *(58)*, and this is dependent on the presence of its C-terminal domain. The other components of a putative ERK7 cascade are not yet known.

2. Properties of the ERK Cascade

The ERK cascade was the first MAPK cascade elucidated *(2)* and has been very extensively studied over the past decade. Several properties of the cascade are described here as a prototype of all MAPK signaling cascades. As mentioned above, the ERK cascade is composed of up to six tiers of sequentially activated protein kinases, which allow amplification and regulation of the transmitted signals. The most important regulatory step in the cascade is the activation of ERKs by MEKs. This process seems to be responsible for the specificity of the cascade and for its impressive cooperativity. This regulation

is made possible by the unique structure and characteristics of the two kinases involved, which are described next.

2.1. Properties of MEKs

There are three members in the MEK family (reviewed in **ref. 59**), MEK1 (45 kDa), MEK2 (46 kDa), and MEK1b (43 kDa). The mechanism of MEK1 activation involves protein phosphorylation on Serines 218 and 222 within its activation loop. Indeed, Alessi et al. *(24)* were able to show that these two Serine residues are phosphorylated by Raf-1 in vitro. The mutation of these and other Ser residues in this region was used *(24,60,61)* to determine that the phosphorylation of both Ser218 and Ser222 is important for full MEK1 activity. Phosphorylation of each one of these residues individually is sufficient to cause partial activation, although Ser222 probably plays a bigger role in this activation *(62)*.

MEKs are highly selective protein kinases that display a high specificity toward the native form of ERKs. Numerous proteins and peptides have been tested, without success, as possible candidates for MEK phosphorylation under conditions that allowed stoichiometric phosphorylation of ERKs *(25)*. Moreover, MEKs failed to recognize either the denatured form of its substrates or peptides containing the phosphorylation sites in ERKs, indicating that the enzyme requires the native form of MAPK. MEKs are also unique in their ability to phosphorylate by themselves both regulatory Thr and Tyr residues of ERKs. Thus, they belong to the small family of dual-specificity protein kinases that also includes the downstream substrates ERK1 and ERK2 *(63)*. However, MEKs and the other MAPKKs are among the very few protein kinases known thus far whose dual specificity has a physiologic function. Phosphorylation of the two residues seems to be a sequential reaction in which Tyr phosphorylation (Tyr185 in ERK2) proceeds Thr183 phosphorylation *(64)*. MEK1b *(25)* does not undergo autophosphorylation and does not have ERK-activating activity *(65)*, raising the question as to what may be its physiologic role. The unique specificity toward the native forms of ERKs *(25)* suggests that MEKs provide specificity as well as an amplification step to the ERK cascade, which singles it out as a central regulatory component in mitogenic signaling pathways.

Beside the activation loop of MEKs, the most important regulatory domain is located in its NH2-terminal region that contains 73 amino acids in MEK1 *(66,67)*. This part of the molecule functions in the regulation of the ERK cascade in several ways. So far it has been shown to contain a nuclear export signal (NES) *(68,69)*, and an ERK-binding region (residues 3–5 in the N-terminus of MEK *[70]*). The NH2-terminal region is also required for efficient feedback phosphorylation by ERK2 in vitro *(71)*; since deletion of the site of interaction in MEK1 reduced the rate of phosphorylation of MEK1 by ERK2

on Ser386. Deletion of this region from MEK1 also reduced its ability to phosphorylate ERK2 in vitro and to stimulate ERK1 and ERK2 in transfected cells *(71)*. Other regulatory sequences in MEKs are the proline-rich regions, which are required for efficient activation of the ERKs *(72)* and probably also for its downregulation *(73)*. These regulatory regions of MEKs provide specificity, amplification, and cooperativity to the whole ERK cascade.

2.2. Properties of ERKs

Three protein kinases were reported to exist in the extensively studied group of ERK/MAPKs (reviewed in **ref. 2**)—ERK1 (p44^{MAPK}); ERK2 (p42^{MAPK}); and ERK1b, which is an alternative spliced form of ERK1 with a molecular mass of 46 kDa *(74)*. Another alternative spliced form of ERK2 was reported at the mRNA level, although the corresponding protein has not yet been identified *(75)*. Common to this group is the signature motif Thr-Glu-Tyr, located in the activation loop. Interestingly, the 110-kDa BMK1 and the 60-kDa ERK7/8 have the Thr-Glu-Tyr motif, but they cannot be activated by MEKs, have a lower degree of similarity to ERK1 and ERK2, and therefore belong to a distinct group of MAPKs. Another protein kinase, termed ERK3 *(10)*, possesses as much as 50% identity to ERK1 and ERK2. However, since this protein has no Thr-Xaa-Tyr motif, it cannot be considered a bona fide MAPK. Because of the high degree of similarity between ERK1 and ERK2, they are usually considered to be functionally redundant, although some differences in their substrate specificity have been reported *(2)*. These isoforms can be activated in response to a wide variety of growth factors and mitogens *(1)*. Activation of these kinases occurs as a result of phosphorylation of the Thr and Tyr residues in a Thr-Xaa-Tyr signature motif. The only upstream mechanism leading to the phosphorylation of ERKs on both of these regulatory residues is their phosphorylation by MEKs. One of the parameters that secures the specificity of MEKs to ERKs is the association between these proteins *(76)*, and ERK was reported to interact also with several other proteins, as described next.

The ERKs are "proline-directed" protein kinases, meaning that they phosphorylate Ser or Thr residues that are neighbors of prolines. Pro-Leu-Ser/Thr-Pro is the most stringent consensus sequence for substrate recognition by ERKs *(77)*. However, the sequence Ser/Thr-Pro can be recognized as well, and the phosphorylation of tyrosine hydroxylase at Ser31 occurs without neighboring prolines *(78)*. Because of the rather broad nature of their substrate recognition, the ERKs can phosphorylate numerous proteins and induce their activation. The main substrates identified thus far are the downstream kinases RSK, MNK, and MSK; the transcription factor Elk-1; the cytosolic PLA$_2$; a few cytoskeletal elements; as well as others *(79)*.

2.3. Structure of ERK2

Activation of protein Ser/Thr kinases by phosphorylation of residues located between their subdomains VII and VIII (i.e., in their activation loop) is the main manner by which signals are transmitted via MAPK cascades. Studies of the mechanism of ERK2 activation *(8)* revealed that both local and global conformational changes of ERK2 are involved in its activation. Like other protein kinases, ERK2 consists of a smaller N-terminal domain made up largely of β strands, and a larger C-terminal domain made up largely of α-helices. The domains are connected by a linker region that allows them to move with respect to each other, while retaining their overall structure. Adenosine triphosphate (ATP) binds in a deep pocket at the interface of the two domains; protein substrates bind on the surface. A surface loop (L12), called the activation loop or phosphorylation lip, contains the Thr183 and Tyr185 phosphorylation sites and lies at the mouth of the active site. Phosphorylation of the Tyr and Thr residues causes a depression in the surface of the substrate binding site of ERK2, thus forming a pocket suitable for positioning the Ser or Thr residue of substrates toward the γ-phosphate of ATP. These changes induce full catalytic activity (~5 μmol/[min·mg]) of ERK2, which is five to six orders of magnitude higher than its basal activity. The three-dimensional structure of unphosphorylated ERK2 and ERK2 mutants, along with the structure of phosphorylated ERK2 *(8,80)*, demonstrates that several segments with low stability in the unphosphorylated enzyme, including the phosphorylation lip and L16, a C-terminal extension to the catalytic core, are positioned differently in the active, phosphorylated structure. In the low-activity state, unphosphorylated Tyr185 partially blocks the protein substrate binding site. In the active state, this phosphorylated residue binds to an anion-binding pocket made up of Arg189 and Arg192, and helps to form the binding surface for the proline following the phosphorylation site in the protein substrate.

2.4. Structure-Function Relationships of ERKs

As mentioned above, a most important regulatory domain in ERKs is their activation loop, whose conformational change on activation not only promotes activation of ERKs, but also induces their detachment from MEKs *(81)*. Interestingly, the region of the activation loop joins a list of several other regions of ERKs that were postulated to be important in the association between ERKs and MEKs. These are residues in subdomain III of ERKs *(82)*; multiple regions in the N- and C-termini of ERKs *(83)*; amino acids 19–25 of ERK2 *(84)*; and residues 312–320 *(85)*, among which residues 316 and 319 *(70)* seem to play the most important role in the interaction with MEKs. It is clear that all these residues cannot interact with a single molecule of MEK1 at the same time, because they are located in completely different areas of the ERK2 molecule.

It is possible, however, that two types of interactions between ERK2 and MEK1 exist. One of these interactions is probably required for the immediate activation of ERK2 by MEK1 and could involve the regions in the same plane of the activation loop *(83)*. The other interaction may involve the cytosolic retention sequence (CRS, also termed *common docking domain* or CD), which does not seem to play a significant role in the activation process of ERK2 *(70,85)*. Although there is accumulating evidence that ERKs and MEKs can directly interact with each other *(76,86)*, it is still possible that this interaction occurs via a third protein such as MP1 for ERK1 *(87)*. In this case, the stimulation-dependent dissociation observed in biochemical experiments *(81)* would not be from MEK1 itself, but from this putative scaffolding protein.

Besides the association with MEKs, ERKs were reported to interact with several other regulatory proteins. Thus, the CRS (CD) of ERKs, which is similar to that of other MAPKs, was implicated in the binding of phosphatases including MAPK phosphatases (MKPs) *(70)* and protein Tyr phosphatases (PTPs). This region also binds downstream substrates of ERKs such as Elk-1 and RSK and apparently increase the specificity of the ERKs to these substrates. Interestingly, abrogation of the CRS significantly gave rise to two naturally occurring isoforms of ERKs, which were regulated differently from the rest of the ERKs under various conditions. One such isoform has been identified in Drosophila in which the analog of Asp339 of ERK1 was mutated to Asn to give rise to a gain-of-function mutant sevenmaker (rl^{sm} *[88]*). In addition, an alternative spliced form of ERK1 with a 26 amino acid insertion just within the CRS has been identified in mammals and termed ERK1b *(74)*. Recent studies demonstrated that this isoform is distinct from that of ERK1 and ERK2 in several aspects. Sensitivity to phosphatases, subcellular localization, substrate specificity, and interaction with MEKs were among the differences between ERK1b and the other ERKs. These parameters lead to a different downregulation of ERK1b as well as different subcellular localization but do not seem to interfere much with the activation processes of ERK1b by MEKs (data not shown). These results indicate again that ERKs' activation does not require a direct interaction with MEKs, which is probably important for the subcellular localization of the ERKs.

Another region of ERK that participates in its protein-protein interaction is loop L6 (residues 91–95), which seems to be important for binding of the ERK molecules to microtubules and other cytoskeletal elements *(89)*. Upon stimulation most of the ERK molecules translocate into the nucleus, but 10–30% of the molecules are activated on the cytoskeletal elements and never detach from it *(90)*. This binding seems to play a role in an ERK2-dependent inhibition of the cytoskeleton organization upon stimulation and involves control of the orientation of actin and the positioning of focal adhesions. Note that, despite the

large number of protein-protein interactions reported for ERK, it still behaves as a monomer under many conditions. This raises the question as to what might be the physiologic relevance of these interactions, which is a point that should be further investigated.

3. Regulation of the ERK Cascade

One of the important considerations in determining MAPK specificity is the strength and duration of the signals. Inactivation of ERKs usually occurs by dephosphorylation and may proceed by the removal of phosphates from Tyr alone, Thr alone, or both residues together. In fact, Tyr phosphatases, Ser/Thr phosphatases, or dual-specificity phosphatases (MKPs) have been implicated in the inactivation of ERKs *(91)*. Moreover, it was shown that exposure to proper phosphatases is an essential step in the regulation of the MAPK cascades *(92)*. The mechanisms of ERK regulation by phosphatases are described next.

3.1. Inactivation of ERKs and MEKs

Inactivation of MAPKs is a very important step in the regulation of biologic outcome of transmitted signals. Since the dual phosphorylation on Thr and Tyr is required to activate a MAPK, both Thr phosphatases and Tyr phosphatases can efficiently inactivate MAPKs. Members of the MKPs are dual-specificity phosphatases, which dephosphorylate Tyr and Thr, in the activation loop of MAPKs. To date, at least nine members of this family have been identified. All possess a characteristic extended active site motif VXVHCXXGXSRSXTXXXAY(L/I)M and N-terminal sequences homolog to the Cdc25 phosphatase. Individual MKPs are selective toward different MAPKs. Among them, MKP1 can dephosphorylate ERKs, JNKs, and p38MAPKs *(93)*, whereas MKP3 is highly selective for ERKs *(94)*. Furthermore, ERK2 was found to associate with MKP3 and cause substrate-triggered activation of MKP3, which results in its inactivation (reviewed in **ref. 95**). Some MKPs (MKP1, MKP2, PAC1, and B23) are localized in the nucleus; however, MKP3 is localized in the cytosol, and localization of M3/6 may change between the nucleus and cytosol in different cell types. The distinct distribution of various MKPs enables cells to differentially regulate MAPKs within different subcellular compartments. All MKPs known to date are inducible proteins and some are immediate early gene products. Their expression is tightly regulated in response to growth and differentiation factors or cellular stresses. For example, NGF stimulation can initiate MKP3 expression in PC12 cells, whereas serum stimulation can only weakly induce this expression *(95)*. Since MKPs do not seem to be significantly expressed in resting cells, it is unlikely that these phosphatases participate in the short-term dephosphorylation of the MAPKs on external stimulation. Taking into account the early phase of

MAPKs inactivation, there are few other possible candidates including the protein Ser/Thr phosphatase and certain PTPs. Two lines of evidence suggest that the Tyr phosphatases PTP-SL, STEP, and HePTP, all of which are structurally related, are major regulators of ERKs *(96)*. First, they physically associate with ERKs through a 16 amino acid kinase interaction motif, located in their cytosolic noncatalytic regions, and subsequently dephosphorylate ERKs *(96–98)*. Second, related to these PTPs, the Drosophila PTP-ER has been genetically shown to inactivate the Drosophila ERK *(99)*. Furthermore, in a mutant PTP-ER strain of Drosophila, the Ras1 signaling pathway is enhanced, resulting in vivo in a MAPK-dependent differentiation of extra R7 neurons *(99)*. Recently, it was shown that inactivation of ERKs in the early stages of mitogenic stimulation involves Tyr phosphatases in the cytosol and a Thr phosphatase in the nucleus *(100)*. Thus, ERKs are differentially regulated in various subcellular compartments to secure proper length and strength of activation, which eventually determines the physiologic outcome of many external signals.

3.2. Substrates of ERKs

The substrates of ERK1 and ERK2 originate in several cellular compartments. Among the various substrates, some are localized in the cytosol, others are cytoskeletal substrates, and there is a group of substrates that resides in the nucleus (for a review *see* **ref. 4**). Thus, the nuclear transcription factor Elk-1 is a well-known substrate for ERK1 and ERK2. Elk-1 is a member of the p62TCF family of transcription factors, which includes additional Ets-related factors such as SAP1 and SAP2. The C-terminal regulatory region of each TCF contains multiple copies of MAPK core consensus for phosphorylation (S/T-P; *[101]*), which is phosphorylated not only by ERKs but also by JNKs and other kinases of the family *(102)*. In the Fos promotor-enhancer, Elk-1 regulates transcription at the serum response element and through its interaction with the serum response factor *(103)*. Most important, Elk-1 is phosphorylated by ERK2 at multiple Ser/Thr-Pro sites *(101)*, and transactivation is potentiated as a result of this phosphorylation *(104–106)*. Other candidate substrates found in the nucleus are transcription modulators, among which are the Ets1, Ets2, and Ets transrepressors *(107)*. In vitro, the transcription modulators Fos, Fra1, and Fra2 are potential targets for direct phosphorylation by ERKs *(4)*. In the cytosol, ERKs have been shown to phosphorylate additional kinases that may further transmit the signals to target molecules such as the RSK, MSK, and MNK. Another substrate at this location is cytosolic PLA_2, the rate-limiting enzyme in pathways involving arachidonic acid release. Phosphorylation on Ser505 results in an increase in its enzymatic activity *(108)*. In the cytoskeleton, ERKs phosphorylate in vitro the proteins Tau, MAP-2, synapsin I, and paxillin *(109)*. An additional set of substrates for the ERKs are upstream proteins of the MAPK

cascade such as growth factor (GF) receptors, SOS, Raf-1, and MEKs, and the list of substrates is still expanding *(79)*. Therefore, it is possible that phosphorylation by ERKs serves as a feedback mechanism for the upstream components that lead to their activation.

3.3. Determination of Specificity of the ERK Cascade

The different types of physiologic functions regulated by the ERK cascade raise the question: What actually determines the specificity of the ERK signals? One such ERK-dependent interplay between two distinct functions downstream of the ERK cascade was observed in PC12 cells *(110)*. In this cell line, stimulation by NGF results in sustained activation of ERKs, which leads to differentiation into cells containing developed neurites *(111)*. On the other hand, EGF leads to a transient activation of ERKs, and the cells subsequently undergo proliferation. Thus, the duration and strength of signals may determine the specificity of the extracellular signals mediated via the ERK cascade. Later it was shown that the sustained activation of ERKs by NGF is mediated by two distinct pathways: first, SOS induces activation of Ras and Raf-1; second, Rap-1 is activated and induces the activation of B-Raf to allow the later stage of ERK activation *(112)*. EGF is unable to induce Rap-1 activation, and therefore, the transient activation is mediated only by the Ras-Raf-1 pathway *(113)*.

In addition to the duration and strength of the signals in the ERK cascade, which is mainly regulated by the upstream machinery and phosphatases of the cascade, other mechanisms contribute to the specificity of extracellular signals. First, as already mentioned, the ERK cascade does not operate alone and is in fact part of a large, multidimensional signaling network, with many inputs to and from other signaling components *(19)*. Although the activity of ERKs is an important factor in determining the outcome of the extracellular signals in these cells, other signaling pathways such as phospholipase Cγ /protein kinase C (PKC), phosphatidylinositol 3'-kinase (PI3K)/PKB, Src/Myc, JNK, and p38MAPK also function simultaneously with the ERK cascade to stimulate certain downstream effects *(19)*. As mentioned above, such additive effects were shown for Elk-1 that can be activated by all known MAPK cascades *(102)*, and recently it was also shown that PLA$_2$ activation by FcγRII in human neutrophils is simultaneously mediated both by ERKs and by p38MAPK *(114)*.

A third mechanism that contributes to the specificity of MAPK signaling is compartmentalization, primarily by scaffold proteins that create multienzyme complexes. The best example of such a mechanism is STE5 in *Saccharomyces cerevisiae*, which governs the activity of the STE11/STE7/FUS3 MAPK cascade and directs its signal to the transcription factor STE12 *(115)*. However, putative MAPK-scaffold proteins have been identified also in mammals *(5,41)*; these proteins facilitate MAPK activation in response to specific extracellular

stimuli, and protect the bound MAPK cascade from irrelevant signals. Interestingly, a putative scaffold, MP1, has been identified as the protein that binds both ERK1 and MEK1 *(87)*. However, in several cells that we examined, this scaffold does not seem to interact with more than a few percent of the ERK1 molecules, and, therefore, MP1 probably plays a leading role in a specified subset of the ERK cascade functions.

Distinct isoforms in the various tiers of each cascade provide an additional mode by which signaling specificity can be achieved. Thus, three components belong to the ERK subfamily of MAPKs, and although they demonstrate a high degree of similarity among themselves, there are still conditions in which these isoforms behave differently. Similarly, many alternatively spliced forms were shown to exist for several components in the MAPK signaling *(74)*. Six alternative spliced forms were identified for the MKK7 *(116)*, five for p38MAPK (e.g., Mxi2; *[117]*), and at least three for BMK1 **(118)**. Although the exact role of all these isoforms is not yet clear, they most probably contribute an additional level of complexity to the network of interacting proteins.

3.4. Subcellular Localization of ERKs and MEKs

Among the key steps in the signaling mechanism of the MAPK cascades are the changes in subcellular localization of their components on extracellular stimulation. In resting cells, all components of the MAPK cascades are apparently localized primarily in the cell cytosol. However, this localization rapidly changes upon extracellular stimulation to allow the transmission of the signals. In the ERK cascade, extracellular stimulation induces Raf1 recruitment to the plasma membrane *(119)* and translocation of MEKs, ERKs, and RSK *(120)* into the nucleus. Correlative and direct evidence indicate that certain functions of ERKs and RSK are completely dependent on their appropriate subcellular localization. Prevention of the nuclear translocation of ERKs strongly inhibited gene transcription, and RSK2 activity in the nucleus was found necessary for EGF-induced transcription of c-fos gene (reviewed in **ref. *4***). Upon stimulation, up to 75% of the ERK molecules translocate and accumulate in the nucleus. In most systems, this accumulation is prolonged, and a large amount of ERKs can be observed in the nucleus long after the ERK activity has declined. For example, in Rat-1 cells, the maximal activity of ERKs in the nucleus is observed within 15 min after EGF application, and this activity rapidly declines owing to dephosphorylation of phosphothreonine, which precedes that of the phosphotyrosine *(100,121)*. Interestingly, the amount of nuclear ERKs (both active and inactive) peaks only 30 min after EGF stimulation, and a large amount of inactive ERKs is observed at this location after more than 60 min. The role of the accumulated inactive ERKs in the nucleus for such a long time and the mechanism that allows this accumulation are not yet clear.

In contrast to ERKs and p90 RSK, it had been suggested that their upstream regulator, MEK1, is absent from the nucleus both prior to and on extracellular stimulation *(122)*. The cytosolic localization of MEK1 is homogeneous, and unlike ERKs, it does not associate with cytoskeletal elements. This subcellular distribution might be important for the activation of MEK1 by its membrane-associated, upstream activator, Raf1. Indeed, it was found that MEK1 contains a short amino acid sequence in the N-terminal region, which acts as an NES, and thus is probably required for cytosolic localization of MEK1 *(68,69)*.

Although it is clear that the proper localization of these kinases is essential for their mitogen-induced functions, the mechanisms regulating the subcellular localization of these enzymes are not fully understood. It was shown that in resting cells ERK2 is retained in the cytosol by its association with MEK1 *(76,85)*, and upon stimulation ERK2 is detached from this cytosolic anchor to rapidly translocate into the nucleus. This study further supported the cytosolic retention of mammalian ERK2 by MEK1, which is reversed on stimulation. As mentioned above, ERKs can be irreversibly retained in the cytosol by MKP3, and several cytoskeletal components as microtubules. Once ERK2 is released from MEK1, no additional signal is required for its translocation into the nucleus, suggesting that the release of ERK2 from MEK1 is the key step in its translocation into the nucleus *(85)*. Interestingly, MEK1 translocates into the nucleus upon stimulation, but is rapidly exported back to the cytosol. The role of MEKs in the nucleus is still unclear, but it has been suggested that it might regulate the activity of ERK1b *(74)*, and that its export serves as a mechanism for the export of ERKs from the nucleus *(123)*.

4. Physiologic Role of the ERK Cascade

Although activation of the ERK cascade was initially implicated in the transmission and control of mitogenic signals, this cascade is now known to be important for differentiation, development, stress response, learning, and morphology determination, discussed next.

4.1. ERKs in Proliferation and Oncogenesis

The rapid activation of MEKs and ERKs in response to mitogens in various cell lines has implicated these kinases in the control of cell proliferation. Moreover, as soon as the ERK cascade had been elucidated, it was noticed that it may participate in the transmission of many mitogenic and oncogenic signals that lead to the accelerated proliferation observed upon malignant transformation. Among the more than 100 oncogenes that are known to date *(124)*, most have been proven to encode proteins that participate in the cascade of events by which growth factors stimulate normal cell division *(125)*. For each level of the growth factor signaling pathways, oncogene homologs have been identi-

fied, and these can be divided into four main classes: growth factors, growth factor receptors, transducers of growth factor responses, and transcription factors. Interestingly, members of the first three groups of oncogenes encode proteins that transmit signals through the ERK cascade, and that their expression causes constitutive activation of ERKs. Members of the fourth group are often located downstream of ERKs *(124)*.

The ERK cascade has been directly implicated in the induction of proliferation and in oncogenic transformation. This was shown by several lines of evidence, including the fact that MAPK activity is stimulated during Ras-mediated transformation *(126)*, by the inhibition of proliferation and oncogenesis by MAPK-specific phosphatase (MKP1; *[127]*), and through the use of the antisense construct of ERK1 *(128)*. However, one of the most convincing lines of evidence for the involvement of the ERK cascade in proliferation was achieved by several investigators *(60,62,129)*, using constitutively active and dominant-negative forms of MEK1. Whereas the dominant-negative form of MEK1 could reverse Ras-mediated transformation, the constitutively activated form served as an oncogene, suggesting that the ERK cascade itself could be sufficient to induce transformation of immortalized cells. A small constitutive activation of ERK1 and ERK2 was observed in 50 tumor cell lines. Cell lines derived from pancreas, colon, lung, ovary, and kidney showed especially high frequencies of constitutive MAPK activation *(130)*. Interestingly, a specific inhibitor of MEKs has been developed (PD184352 *[131]*) and shown to inhibit tumor growth as much as 80% in mice with colon carcinomas of both mouse and human origin. Since activation of the ERK cascade participates in many types of malignancies, this inhibitor may serve as a general tool in combating cancer.

4.2. ERKs in Cell-Cycle Control

Sustained, as opposed to transient, activation of ERKs appears to be required for many cells to pass the G1 restriction point and to enter the S-phase, in which cellular DNA is replicated *(128)*. Although ERK activation is linked to the cell cycle, it had not been clear where the ERK pathway might interact with the cell-cycle machinery. Expression of the D-type cyclins, which are the regulatory subunits for the cyclin-dependent kinase 4 (CDK4) and CDK6 catalytic subunits, controls the early stages of the transition toward the S-phase. A critical link between signal transduction and the cell cycle has been suggested by the finding that the expression of dominant inhibitory mutants of MEKs and ERKs or the expression of MKP1 inhibited the growth factor-dependent expression of cyclin D1. The expression of constitutively active mutants of MEKs or various Raf constructs increased cyclin D1 expression *(132,133)*. Although the ERK cascade appears to be required for growth factor signaling in order to activate cyclin D1 expression in a variety of systems, clearly, this is

not the only signaling pathway required. Activation of the PI3K pathway is also required, since activation of ERKs resulting from inducible MEK constructs resulted in cyclin D1 expression only when a PI3-kinase signal was present *(134)*. In addition to the regulation of Cdk4 and Cdk6 activity through the synthesis of D-type cyclins, the cell-cycle machinery is regulated by the Cdk inhibitors (CKIs). Degradation of CKI p27^{Kip1} appears to be an important control point for entry into the cell cycle and may be a key regulator of cyclin E/Cdk2 activity. In vitro, p27^{Kip1} can be phosphorylated by ERKs, although the actual sites of phosphorylation have not been identified and it has been argued that the ERK pathway is involved in the degradation of p27^{Kip1} *(135)*. A direct role for ERKs activity was shown by experiments in which activation of ERKs in MEK-inducible cell lines led to p27^{Kip1} degradation many hours before any cyclin E/Cdk2 activity was measurable *(134)*. Whereas low levels of activated Raf cause cell-cycle progression, high levels cause cell-cycle arrest and p21$^{waf1/Cip1}$ induction in a p53-independent manner *(136)*. In addition, activated Ras or sustained activation of ERKs accelerate the onset of senescence in some cells *(137)* and induce growth arrest in others *(133,136,138)*. Moreover, there is evidence of cross talk between the proliferation/differentiation pathways activated by the ERK cascade and the growth arrest functions of tumor suppressor genes including *p53*, *p16*, and *Rb (139)*. Thus, sustained Ras or Raf signaling was reported to activate p53 or p21 or both, as well as p16 expression, leading to growth arrest *(140,141)*.

In addition to the known role of MEK1 in cell-cycle entry from G0, the level of MEK1 activity affected the kinetics of progression through both the G1- and G2-phases of the cell cycle in NIH-3T3 cells. Ectopic expression of dominant-negative forms of MEK1, which was previously shown to inhibit G0/G1 progression, was also found to delay the progression of cells through G2. In addition, treatment of cells with an MEK1 inhibitor during a synchronous S-phase arrested the cells in the following G2-phase *(142)*. Recently, MEK1 was shown to specifically undergo activation by phosphorylation during mitosis *(143)*. This activation is required for fragmentation of the pericentriolarly organized Golgi apparatus. Surprisingly, the cytosolic downstream targets of MEK1, ERK1, and ERK2 do not seem to be required for MEK1-dependent Golgi fragmentation *(144)*.

4.3. ERKs in Other Physiologic Processes

Another physiologic response that appears to be regulated through the MAPK signaling cascades is cellular differentiation. Different members of the MAPK cascades have been implicated in processes such as monocytic differentiation *(145)*, neurite outgrowth of PC12 cells *(146)*, T-cell maturation *(147)*, and mast cell development *(148)*. Since ERKs are activated in somatic cells in

response to many extracellular stimuli, it is not surprising that ERKs are also involved in developmental processes required for proliferation of a new group of cells when new organs develop in the growing organisms. Indeed, such involvement has been clearly demonstrated in several developmental systems such as in *Drosophila* embryogenesis *(149,150)*, *Xenopus* embryogenesis *(151)*, and in *Caenorhabditis elegans* vulval development *(152)*.

In most cell types and conditions, the ERK cascade seems to play an antiapoptotic effect, and a reduction in its activity is essential for the process of apoptosis to proceed. Thus, it was shown that in serum-starved PC12 cells ERK cascade is inhibited in correlation to cell death *(153)*. Moreover, activation of the ERK cascade protects NIH3T3 cells against doxorubicin-induced cell death *(154)*. Prevention of apoptosis by the ERK cascade can occur by Raf1, which is involved in the phosphorylation of the mitochondrial protein Bad, thereby preventing its interaction with Bcl-2 and inhibiting apoptosis. This Raf-induced protection from apoptosis involves activation of MEK, ERK, and RSK *(155)*. On the other hand, the ERK cascade was also shown to be involved in the induction of apoptosis in some systems. *De novo*-synthesized ceramide signals apoptosis in astrocytes via the ERK cascade *(156)*, and the ERK cascade also plays a role in apoptosis caused by taxol *(18)*. Therefore, although a rare event, the ERK cascade may be involved in the onset of apoptosis in some cellular systems.

Activation of ERK1 and ERK2 has also been implicated in synaptic plasticity and memory. Processes of learning and memory in mammalian brains involve the establishment of new synaptic connections, and these are regulated by several intracellular signaling pathways. The involvement of ERKs in learning have been demonstrated in aplysia *(157)* as well as in several model systems such as taste-learning, fear condition, and the acquisition of memory *(158)*. ERKs probably play some role in additional cellular processes such as morphology determination, migration, stress response immunologic reactions, and cell survival. However, in some of the processes, the role of ERKs is only secondary and may be cell type specific.

In summary, we have described here the ERK cascade that serves as a central signaling vehicle from the plasma membrane to intracellular target molecules, and thus control various activities evoked by growth factors and other extracellular stimuli. The ERK cascade was the first MAPK cascade to be elucidated and, together with the JNK, p38MAPK, BMK1, and ERK7 MAPK cascades, forms a complex network of interacting proteins that govern most stimulated physiologic processes. The ERK cascade is composed of four to six tiers of sequentially activated protein kinases, and among them Raf-1, MEKs, and ERKs are the core components of mitogenic stimulation. The activation of each of the protein kinases in the cascade occurs by phosphorylation, and in

many of the components this phosphorylation occurs on residues in their activation loop. This review has described the role of the ERK cascade in a wide variety of cellular processes such as proliferation, differentiation, development, and cell cycle. This book describes the methods used in the study of MAPK signaling in many of these systems.

References

1. Campbell, J. S., Seger, R., Graves, J. D., Graves, L. M., Jensen, A. M., and Krebs, E. G. (1995) The MAP kinase cascade. *Recent Prog. Horm. Res.* **50,** 131–159.
2. Seger, R. and Krebs, E. G. (1995) The MAPK signaling cascade. *FASEB J.* **9,** 726–735.
3. Rubinfeld, H. and Seger, R. (1998) Detection of MAPK cascades. *Curr. Protocols Cell Biol.* **14.3,** 1–19.
4. Lewis, T. S., Shapiro, P. S., and Ahn, N. G. (1998) Signal transduction through MAP kinase cascades. *Adv. Cancer Res.* **74,** 49–139.
5. Schaeffer, H. J. and Weber, M. J. (1999) Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol. Cell. Biol.* **19,** 2435–2444.
6. Chang, L. and Karin, M. (2001) Mammalian MAP kinase signalling cascades. *Nature* **410,** 37–40.
7. Pearson, G., Robinson, F., Beers Gibson, T., et al. (2001) Mitogen-activated protein (map) kinase pathways: regulation and physiological functions. *Endocr. Rev.* **22,** 153–183.
8. Canagarajah, B. J., Khokhlatchev, A., Cobb, M. H., and Goldsmith, E. J. (1997) Activation mechanism of the MAP kinase ERK2 by dual phosphorylation. *Cell* **90,** 859–869.
9. Payne, D. M., Rossomando, A. J., Martino, P., et al. (1991) Identification of the regulatory phosphorylation sites in pp42/mitogen activated protein kinase (MAP kinase). *EMBO J.* **10,** 885–892.
10. Boulton, T. G., Nye, S. H., Robbins, D. J., et al. (1991) ERK's: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell* **65,** 663–675.
11. Derijard, B., Hibi, M., Wu, I. H., et al. (1994) JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* **76,** 1025–1027.
12. Kyriakis, J. M., Banerjee, P., Nikolakaki, E., et al. (1994) The stress-activated protein kinase subfamily of c-Jun kinases. *Nature* **369,** 156–160.
13. Freshney, N. W., Rawlinson, L., Guesdon, F., et al. (1994) Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of Hsp27. *Cell* **78,** 1039–1049.
14. Han, J., Lee, J. D., Bibbs, L., and Ulevitch, R. J. (1994) A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* **265,** 808–811.

15. Rouse, J., Cohen, P., Trigon, S., et al. (1994) A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell* **78,** 1027–1037.
16. Zhou, G., Bao, Z. Q., and Dixon, J. E. (1995) Components of a new human protein kinase signal transduction pathway. *J. Biol. Chem.* **270,** 12,665–12,669.
17. Lee, J. D., Ulevitch, R. J., and Han, J. (1995) Primary structure of BMK1: a new mammalian map kinase. *Biochem. Biophys. Res. Commun.* **213,** 715–724.
18. Bacus, S. S., Gudkov, A. V., Lowe, M., et al. (2001) Taxol-induced apoptosis depends on MAP kinase pathways (ERK and p38) and is independent of p53. *Oncogene* **20,** 147–155.
19. Naor, Z., Benard, O., and Seger, R. (2000) Activation of MAPK cascades by G-protein-coupled receptors: the case of gonadotropin-releasing hormone receptor. *Trends Endocrinol. Metab.* **11,** 91–99.
20. Schlessinger, J. (1993) How receptor tyrosine kinases activate Ras. *Trends Biochem. Sci.* **18,** 273–275.
21. Rane, S. G. (1999) Ion channels as physiological effectors for growth factor receptor and Ras/ERK signaling pathways. *Adv. Second Messenger Phosphoprotein Res.* **33,** 107–127.
22. Sternberg, P. W. and Alberola-Ila, J. (1998) Conspiracy theory: RAS and RAF do not act alone. *Cell* **95,** 447–450.
23. Barkoff, A., Ballantyne, S., and Wickens, M. (1998) Meiotic maturation in Xenopus requires polyadenylation of multiple mRNAs. *EMBO J.* **17,** 3168–3175.
24. Alessi, D. R., Saito, Y., Campbell, D. G., et al. (1994) Identification of the sites in MAP kinase kinase-1 phosphorylated by p74raf-1. *EMBO J.* **13,** 1610–1619.
25. Seger, R., Ahn, N. G., Posada, J., et al. (1992) Purification and characterization of MAP kinase activator(s) from epidermal growth factor stimulated A431 cells. *J. Biol. Chem.* **267,** 14,373–14,381.
26. Sturgill, T. W., Ray, L. B., Erikson, E., and Maller, J. L. (1988) Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. *Nature* **334,** 715–718.
27. Deak, M., Clifton, A. D., Lucocq, L. M., and Alessi, D. R. (1998) Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. *EMBO J.* **17,** 4426–4441.
28. Fukunaga, R. and Hunter, T. (1997) MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. *EMBO J.* **16,** 1921–1933.
29. Waskiewicz, A. J., Flynn, A., Proud, C. G., and Cooper, J. A. (1997) Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. *EMBO J.* **16,** 1909–1920.
30. Eldar-Finkelman, H., Seger, R., Vandenheede, J. R., and Krebs, E. G. (1995) Inactivation of glycogen synthase kinase-3 by epidermal growth factor is mediated by mitogen-activated protein kinase/p90 ribosomal protein S6 kinase signaling pathway in NIH/3T3 cells. *J. Biol. Chem.* **270,** 987–990.

31. Sapkota, G. P., Kieloch, A., Lizcano, J. M., et al. (2001) Phosphorylation of the protein kinase mutated in Peutz-Jeghers cancer syndrome, LKB1/STK11, at Ser431 by p90RSK and cAMP-dependent protein kinase, but not its farnesylation at Cys433, is essential for LKB1 to suppress cell growth. *J. Biol. Chem.* **276,** 19,469–19,482.

32. Kyriakis, J. M. (1999) Making the connection: coupling of stress-activated ERK/ MAPK (extracellular-signal-regulated kinase/mitogen-activated protein kinase) core signalling modules to extracellular stimuli and biological responses. *Biochem. Soc. Symp.* **64,** 29–48.

33. Ono, K. and Han, J. (2000) The p38 signal transduction pathway: activation and function. *Cell Sig.* **12,** 1–13.

34. Lee, J. C., Laydon, J. T., McDonnell, P. C., et al. (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature* **372,** 739–746.

35. Cuenda, A., Cohen, P., Buee-Scherrer, V., and Goedert, M. (1997) Activation of stress-activated protein kinase-3 (SAPK3) by cytokines and cellular stresses is mediated via SAPKK3 (MKK6): comparison of the specificities of SAPK3 and SAPK2 (RK/p38). *EMBO J.* **16,** 295–305.

36. Goedert, M., Cuenda, A., Craxton, M., Jakes, R., and Cohen, P. (1997) Activation of the novel stress-activated protein kinase SAPK4 by cytokines and cellular stresses is mediated by SKK3 (MKK6); comparison of its substrate specificity with that of other SAP kinases. *EMBO J.* **16,** 3563–3571.

37. Stokoe, D., Campbell, D. G., Nakielny, S., et al. (1992) MAPKAP kinase-2: a novel protein kinase activated by mitogen-activated protein kinase. *EMBO J.* **11,** 3985–3994.

38. McLaughlin, M. M., Kumar, S., McDonnell, P. C., et al. (1996) Identification of mitogen-activated protein (MAP) kinase-activated protein kinase-3, a novel substrate of CSBP p38 MAP kinase. *J. Biol. Chem.* **271,** 8488–8492.

39. New, L., Jiang, Y., Zhao, M., et al. (1998) PRAK, a novel protein kinase regulated by the p38 MAP kinase. *EMBO J.* **17,** 3372–3384.

40. Kramer, R. M., Roberts, E. F., Um, S. L., et al. (1996) p38 mitogen-activated protein kinase phosphorylates cytosolic phospholipase A2 (cPLA2) in thrombin-stimulated platelets: evidence that proline-directed phosphorylation is not required for mobilization of arachidonic acid by cPLA2. *J. Biol. Chem.* **271,** 27,723–27,729.

41. Davis, R. J. (2000) Signal transduction by the JNK group of MAP kinases. *Cell* **103,** 239–252.

42. Coso, O. A., Chiariello, M., Yu, J. C., et al. (1995) The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell* **81,** 1137–1146.

43. Dan, I., Watanabe, N. M., and Kusumi, A. (2001) The Ste20 group kinases as regulators of MAP kinase cascades. *Trends Cell. Biol.* **11,** 220–230.

44. Yan, M., Dai, T., Deak, J. C., et al. (1994) Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1. *Nature* **372,** 798–800.

45. Holland, P. M., Suzanne, M., Campbell, J. S., Noselli, S., and Cooper, J. A. (1997) MKK7 is a stress-activated mitogen-activated protein kinase kinase functionally related to hemipterous. *J. Biol. Chem.* **272,** 24,994–24,998.

46. Tournier, C., Whitmarsh, A. J., Cavanagh, J., Barrett, T., and Davis, R. J. (1997) Mitogen-activated protein kinase kinase 7 is an activator of the c-Jun NH2-terminal kinase. *Proc. Natl. Acad. Sci. USA* **94,** 7337–7342.

47. Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993) Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes Dev.* **7,** 2135–2148.

48. Deng, X., Xiao, L., Lang, W., et al. (2001) Novel role for JNK as a stress-activated Bcl2 kinase. *J. Biol. Chem.* **276,** 23,681–23,688.

49. Zhang, Y., Zhong, S., Dong, Z., Chen, N., Bode, A. M., and Ma, W. (2001) UVA induces Ser381 phosphorylation of p90RSK/MAPKAP-K1 via ERK and JNK pathways. *J. Biol. Chem.* **276,** 14,572–14,580.

50. Chiariello, M., Marinissen, M. J., and Gutkind, J. S. (2000) Multiple mitogen-activated protein kinase signaling pathways connect the cot oncoprotein to the c-jun promoter and to cellular transformation. *Mol. Cell. Biol.* **20,** 1747–1758.

51. Gotoh, I., Adachi, M., and Nishida, E. (2001) Identification and characterization of a novel map kinase kinase kinase, mltk. *J. Biol. Chem.* **276,** 4276–4286.

52. Chao, T. H., Hayashi, M., Tapping, R. I., Kato, Y., and Lee, J. D. (1999) MEKK3 directly regulates MEK5 activity as part of the big mitogen-activated protein kinase 1 (BMK1) signaling pathway. *J. Biol. Chem.* **274,** 36,035–36,038.

53. Chayama, K., Papst, P. J., Garrington, T. P., et al. (2001) Role of MEKK2-MEK5 in the regulation of TNF-alpha gene expression and MEKK2-MKK7 in the activation of c-Jun N-terminal kinase in mast cells. *Proc. Natl. Acad. Sci. USA* **98,** 4599–4604.

54. Kato, Y., Chao, T. H., Hayashi, M., Tapping, R. I., and Lee, J. D. (2000) Role of BMK1 in regulation of growth factor-induced cellular responses. *Immunol. Res.* **21,** 233–237.

55. Kasler, H. G., Victoria, J., Duramad, O., and Winoto, A. (2000) ERK5 is a novel type of mitogen-activated protein kinase containing a transcriptional activation domain. *Mol. Cell. Biol.* **20,** 8382–8389.

56. Hayashi, M., Tapping, R. I., Chao, T. H., et al. (2001) BMK1 mediates growth factor-induced cell proliferation through direct cellular activation of serum and glucocorticoid-inducible kinase. *J. Biol. Chem.* **276,** 8631–8634.

57. Abe, M. K., Kuo, W. L., Hershenson, M. B., and Rosner, M. R. (1999) Extracellular signal-regulated kinase 7 (ERK7), a novel ERK with a C-terminal domain that regulates its activity, its cellular localization, and cell growth. *Mol. Cell. Biol.* **19,** 1301–1312.

58. Abe, M. K., Kahle, K. T., Saelzler, M. P., Orth, K., Dixon, J. E., and Rosner, M. R. (2001) ERK7 is an autoactivated member of the MAP kinase family. *J. Biol. Chem.* **276,** 21,272–21,279.

59. Seger, R., Biener, Y., Feinstein, R., Hanoch, T., Gazit, A., and Zick, Y. (1995) Differential activation of mitogen-activated protein kinase and S6 kinase sig-

naling pathways by 12-O-tetradecanoylphorbol-13-acetate (TPA) and insulin. Evidence for involvement of a TPA-stimulated protein-tyrosine kinase. *J. Biol. Chem.* **270,** 28,325–28,330.

60. Cowley, S., Paterson, H., Kemp, P., and Marshall, C. J. (1994) Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. *Cell* **77,** 841–852.

61. Zheng, C. F. and Guan, K. L. (1994) Activation of MEK family kinases requires phosphorylation of two conserved Ser/Thr residues. *EMBO J.* **13,** 1123–1131.

62. Seger, R., Seger, D., Reszka, A. A., et al. (1994) Over-expression of mitogen-activated protein kinase kinase (MAPKK) and its mutants in NIH-3T3 cells: evidence that MAPKK's involvement in cellular proliferation is regulated by phosphorylation of serine residues in its kinase subdomains VII and VIII. *J. Biol. Chem.* **269,** 25,699–25,709.

63. Seger, R., Ahn, N. G., Boulton, T. G., et al. (1991) Microtubule-associated protein 2 kinases, ERK1 and ERK2, undergo autophosphorylation on both tyrosine and threonine residues: implication for their mechanism of activation. *Proc. Natl. Acad. Sci. USA* **88,** 6142–6146.

64. Haystead, T. A., Dent, P., Wu, J., Haystead, C. M., and Sturgill, T. W. (1992) Ordered phosphorylation of p42mapk by MAP kinase kinase. *FEBS Lett.* **306,** 17–22.

65. Zheng, C. F. and Guan, K. L. (1993) Properties of MEKs, the kinases that phosphorylate and activate the extracellular signal-regulated kinases. *J. Biol. Chem.* **268,** 23,933–23,939.

66. Crews, C. M., Alessandrini, A., and Erikson, R. L. (1992) The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. *Science* **258,** 478–480.

67. Seger, R., Seger, D., Lozeman, F. J., et al. (1992) Human T-cell Map kinase kinases are related to yeast signal transduction kinases. *J. Biol. Chem.* **267,** 25,628–25,631.

68. Fukuda, M., Gotoh, I., Gotoh, Y., and Nishida, E. (1996) cytoplasmic localization of mitogen-activated protein kinase kinase directed by its NH2-terminal, leucine-rich short amino acid sequence, which acts as a nuclear export signal. *J. Biol. Chem.* **271,** 20,024–20,028.

69. Jaaro, H., Rubinfeld, H., Hanoch, T., and Seger, R. (1997) Nuclear translocation of mitogen-activated protein kinase kinase (MEK1) in response to mitogenic stimulation. *Proc. Natl. Acad. Sci. USA* **94,** 3742–3747.

70. Tanoue, T., Adachi, M., Moriguchi, T., and Nishida, E. (2000) A conserved docking motif in MAP kinases common to substrates, activators and regulators. *Nat. Cell. Biol.* **2,** 110–116.

71. Xu, B., Wilsbacher, J. L., Collisson, T., and Cobb, M. H. (1999) The N-terminal ERK-binding site of MEK1 is required for efficient feedback phosphorylation by ERK2 in vitro and ERK activation in vivo. *J. Biol. Chem.* **274,** 34,029–34,035.

72. Dang, A., Frost, J. A., and Cobb, M. H. (1998) The MEK1 proline-rich insert is required for efficient activation of the mitogen-activated protein kinases ERK1 and ERK2 in mammalian cells. *J. Biol. Chem.* **273,** 19,909–19,913.

73. Rossomando, A. J., Dent, P., Sturgill, T. W., and Marshak, D. R. (1994) Mitogen-activated protein kinase kinase 1 (MKK1) is negatively regulated by threonine phosphorylation. *Mol. Cell Biol.* **14,** 1594–1602.

74. Yung, Y., Yao, Z., Hanoch, T., and Seger, R. (2000) ERK1b: a 46 kD ERK isoform that is differentially regulated by MEK. *J. Biol. Chem.* **275,** 15,799–15,808.

75. Gonzalez, F. A., Raden, D. L., Rigby, M. R., and Davis, R. J. (1992) Heterogeneous expression of four MAP kinase isoforms in human tissues. *FEBS Lett.* **304,** 170–178.

76. Fukuda, M., Gotoh, Y., and Nishida, E. (1997) Interaction of MAP kinase with MAP kinase kinase: its possible role in the control of nucleocytoplasmic transport of MAP kinase. *EMBO J.* **16,** 1901–1908.

77. Gonzalez, F. A., Raden, D. L., and Davis, R. J. (1991) Identification of substrate recognition determinants for human ERK1 and ERK2 protein kinases. *J. Biol. Chem.* **266,** 22,159–22,163.

78. Haycock, J. W., Ahn, N. G., Cobb, M. H., and Krebs, E. G. (1992) ERK1 and ERK2, two microtubule-associated protein 2 kinases, mediate the phosphorylation of tyrosine hydroxylase at serine-31 in situ. *Proc. Natl. Acad. Sci. USA* **89,** 2365–2369.

79. Lewis, T. S., Hunt, J. B., Aveline, L. D., et al. (2000) Identification of novel MAP kinase pathway signaling targets by functional proteomics and mass spectrometry. *Mol. Cell* **6,** 1343–1354.

80. Zhang, F., Strand, A., Robbins, D., Cobb, M. H., and Goldsmith, J. G. (1994) Atomic structure of the MAP kinase ERK2 at 2.3 Å resolution. *Nature* **367,** 704–711.

81. Wolf, I., Rubinfeld, H., Yoon, S., Marmor, G., Hanoch, T., and Seger, R. (2001) Involvement of the activation loop of ERK in the detachment from cytosolic anchoring. *J. Biol. Chem.* **276,** 24,490–24,497.

82. Brunet, A. and Pouyssegur, J. (1996) Identification of MAP kinase domains by redirecting stress signals into growth factor responses. *Science* **272,** 1652–1655.

83. Wilsbacher, J. L., Goldsmith, E. J., and Cobb, M. H. (1999) Phosphorylation of MAP kinases by MAP/ERK involves multiple regions of MAP kinases. *J. Biol. Chem.* **274,** 16,988–16,994.

84. Eblen, S. T., Catling, A. D., Assanah, M. C., and Weber, M. J. (2001) Biochemical and biological functions of the N-terminal, noncatalytic domain of extracellular signal-regulated kinase 2. *Mol. Cell. Biol.* **21,** 249–259.

85. Rubinfeld, H., Hanoch, T., and Seger, R. (1999) Identification of a cytoplasmic-retention sequence in ERK2. *J. Biol. Chem.* **274,** 30,349–30,352.

86. Bardwell, A. J., Flatauer, L. J., Matsukuma, K., Thorner, J., and Bardwell, L. (2001) A conserved docking site in meks mediates high-affinity binding to map kinases and cooperates with a scaffold protein to enhance signal transmission. *J. Biol. Chem.* **276,** 10,374–10,386.

87. Schaeffer, H. J., Catling, A. D., Eblen, S. T., Collier, L. S., Krauss, A., and Weber, M. J. (1998) MP1: a MEK binding partner that enhances enzymatic activation of the MAP kinase cascade. *Science* **281,** 1668–1671.

88. Bott, C. M., Thorneycroft, S. G., and Marshall, C. J. (1994) The sevenmaker gain-of-function mutation in p42 MAP kinase leads to enhanced signalling and reduced sensitivity to dual specificity phosphatase action. *FEBS Lett.* **352,** 201–205.

89. Reszka, A. A., Bulinski, J. C., Krebs, E. G., and Fischer, E. H. (1997) Mitogen-activated protein kinase/extracellular signal-regulated kinase 2 regulates cytoskeletal organization and chemotaxis via catalytic and microtubule-specific interactions. *Mol. Biol. Cell.* **8,** 1219–1232.

90. Reszka, A. A., Seger, R., Diltz, C. D., Krebs, E. G., and Fischer, E. H. (1995) Association of mitogen-activated protein kinase with the microtubule cytoskeleton. *Proc. Natl. Acad. Sci. USA* **92,** 8881–8885.

91. Alessi, D. R., Gomez, N., Moorhead, G., Lewis, T., Keyse, S. M., and Cohen, P. (1995) Inactivation of p42 MAP kinase by protein phosphatase 2A and a protein tyrosine phosphatase, but not CL100, in various cell lines. *Curr. Biol.* **5,** 283–295.

92. Keyse, S. M. (1998) Protein phosphatases and the regulation of MAP kinase activity. *Semin. Cell Dev. Biol.* **9,** 143–152.

93. Chu, Y., Solski, P. A., Khosravi-Far, R., Der, C. J., and Kelly, K. (1996) The mitogen-activated protein kinase phosphatases PAC1, MKP-1, and MKP-2 have unique substrate specificities and reduced activity in vivo toward the ERK2 sevenmaker mutation. *J. Biol. Chem.* **271,** 6497–6501.

94. Muda, M., Theodosiou, A., Rodrigues, N., et al. (1996) The dual specificity phosphatases M3/6 and MKP-3 are highly selective for inactivation of distinct mitogen-activated protein kinases. *J. Biol. Chem.* **271,** 27,205–27,208.

95. Camps, M., Nichols, A., and Arkinstall, S. (2000) Dual specificity phosphatases: a gene family for control of MAP kinase function. *FASEB J.* **14,** 6–16.

96. Pulido, R., Zuniga, A., and Ullrich, A. (1998) PTP-SL and STEP protein tyrosine phosphatases regulate the activation of the extracellular signal-regulated kinases ERK1 and ERK2 by association through a kinase interaction motif. *EMBO J.* **17,** 7337–7350.

97. Oh-hora, M., Ogata, M., Mori, Y., et al. (1999) Direct suppression of TCR-mediated activation of extracellular signal-regulated kinase by leukocyte protein tyrosine phosphatase, a tyrosine-specific phosphatase. *J. Immunol.* **163,** 1282–1288.

98. Zuniga, A., Torres, J., Ubeda, J., and Pulido, R. (1999) Interaction of mitogen-activated protein kinases with the kinase interaction motif of the tyrosine phosphatase PTP-SL provides substrate specificity and retains ERK2 in the cytoplasm. *J. Biol. Chem.* **274,** 21,900–21,907.

99. Karim, F. D. and Rubin, G. M. (1999) PTP-ER, a novel tyrosine phosphatase, functions downstream of Ras1 to downregulate MAP kinase during Drosophila eye development. *Mol. Cell.* **3,** 741–750.

100. Yao, Z., Dolginov, Y., Hanoch, T., et al. (2000) Detection of partially phosphorylated forms of ERK by monoclonal antibodies reveals spatial regulation of ERK activity by phosphatases. *FEBS Lett.* **468,** 37–42.

101. Cruzalegui, F. H., Cano, E., and Treisman, R. (1999) ERK activation induces phosphorylation of Elk-1 at multiple S/T-P motifs to high stoichiometry. *Oncogene* **18,** 7948–7957.

102. Cavigelli, M., Dolfi, F., Claret, F. X., and Karin, M. (1995) Induction of c-fos expression through JNK-mediated TCF/Elk-1 phosphorylation. *EMBO J.* **14,** 5957–5964.
103. Treisman, R. (1994) Ternary complex factors: growth factor regulated transcriptional activators. *Curr. Opin. Genet. Dev.* **4,** 96–101.
104. Gille, H., Sharrocks, A. D., and Shaw, P. E. (1992) Phosphorylation of transcription factor p62TCF by MAP kinase stimulates ternary complex formation at c-fos promoter. *Nature* **358,** 414–417.
105. Marais, R., Wynne, J., and Treisman, R. (1993) The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell* **73,** 381–393.
106. Whitmarsh, A. J., Shore, P., Sharrocks, A. D., and Davis, R. J. (1995) Integration of MAP kinase signal transduction pathways at the serum response element. *Science* **269,** 403–407.
107. Wasylyk, B., Hagman, J., and Gutierrez-Hartmann, A. (1998) Ets transcription factors: nuclear effectors of the Ras-MAP-kinase signaling pathway. *Trends Biochem. Sci.* **23,** 213–216.
108. Lin, L. L., Wartmann, M., Lin, A. Y., Knopf, J. L., Seth, A., and Davis, R. J. (1993) cPLA2 is phosphorylated and activated by MAP kinase. *Cell* **72,** 269–278.
109. Jovanovic, J. N., Benfenati, F., Siow, Y. L., et al. (1996) Neurotrophins stimulate phosphorylation of synapsin I by MAP kinase and regulate synapsin I-actin interactions. *Proc. Natl. Acad. Sci. USA* **93,** 3679–3683.
110. Peraldi, P., Scimeca, J., Filloux, C., and Van Obberghen, E. (1993) Regulation of extracellular signal regulated protein kinase-1 (ERK1; pp44/mitogen-activated protein kinase) by epidermal groeth factor and nerve growth factor in PC12 cells: implication of ERK1 inhibitory activities. *Endocrinology* **132,** 2578–2585.
111. Marshall, C. J. (1995) Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80,** 179–185.
112. York, R. D., Yao, H., Dillon, T., et al. (1998) Rap1 mediates sustained MAP kinase activation induced by nerve growth factor. *Nature* **392,** 622–626.
113. Grewal, S. S., York, R. D., and Stork, P. J. (1999) Extracellular-signal-regulated kinase signalling in neurons. *Curr. Opin. Neurobiol.* **9,** 544–553.
114. Hazan-Halevy, I., Seger, R., and Levy, R. (2000) The requirement of both extracellular regulated kinase and p38 mitogen-activated protein kinase for stimulation of cytosolic phospholipase A(2) activity by either FcgammaRIIA or FcgammaRIIIB in human neutrophils: a possible role for pyk2 but not for the grb2-sos-shc complex. *J. Biol. Chem.* **275,** 12,416–12,423.
115. Elion, E. A. (2000) Pheromone response, mating and cell biology. *Curr. Opin. Microbiol.* **3,** 573–581.
116. Tournier, C., Whitmarsh, A. J., Cavanagh, J., Barrett, T., and Davis, R. J. (1999) The MKK7 gene encodes a group of c-Jun NH2-terminal kinase kinases. *Mol. Cell. Biol.* **19,** 1569–1581.
117. Sanz, V., Arozarena, I., and Crespo, P. (2000) Distinct carboxy-termini confer divergent characteristics to the mitogen-activated protein kinase p38alpha and its splice isoform Mxi2. *FEBS Lett.* **474,** 169–174.

118. Yan, C., Luo, H., Lee, J. D., Abe, J., and Berk, B. C. (2001) Molecular cloning of mouse ERK5/BMK1 splice variants and characterization of ERK5 functional domains. *J. Biol. Chem.* **276,** 10,870–10,878.

119. Leevers, S. J., Paterson, H. F., and Marshall, C. J. (1994) Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. *Nature* **369,** 411–414.

120. Chen, R. H., Sarnecki, C., and Blenis, J. (1992) Nuclear localization and regulation of erk- and rsk-encoded protein kinases. *Mol. Cell. Biol.* **12,** 915–927.

121. Yung, Y., Dolginov, Y., Yao, Z., et al. (1997) Detection of ERK activation by a novel monoclonal antibody. *FEBS Lett.* **408,** 292–296.

122. Lenormand, P., Sardet, C., Pages, G., L'Allemain, G., Brunet, A., and Pouyssegur, J. (1993) Growth factors induce nuclear translocation of MAP kinases (p42mapk and p44mapk) but not of their activator MAP kinase kinase (p45mapkk) in fibroblasts. *J. Cell. Biol.* **122,** 1079–1088.

123. Adachi, M., Fukuda, M., and Nishida, E. (2000) Nuclear export of MAP kinase (ERK) involves a MAP kinase kinase (MEK)-dependent active transport mechanism [published erratum appears in *J. Cell. Biol.* 2000; **149(3),** 754]. *J. Cell. Biol.* **148,** 849–856.

124. Hunter, T. (1991) Cooperation between oncogenes. *Cell* **64,** 249–270.

125. Sherr, C. J. (1996) Cancer cell cycle. *Science* **274,** 1672–1677.

126. Kyriakis, J. M., App, H., Zhang, F. X., et al. (1992) Raf-1 activates MAP kinase-kinase. *Nature* **358,** 417–421.

127. Sun, H., Charles, C. H., Lau, L. F., and Tonks, N. K. (1993) MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase in vivo. *Cell* **75,** 487–493.

128. Pages, G., Lenormand, P., L'Allemain, G., et al. (1993) Mitogen-activated protein kinases p42mapk and p44mapk are required for fibroblast proliferation. *Proc. Natl. Acad. Sci. USA* **90,** 8319–8323.

129. Mansour, S. J., Matten, W. T., Hermann, A. S., et al. (1994) Transformation of mammalian cells by constitutively active MAP kinase kinase. *Science* **265,** 966–970.

130. Hoshino, R., Chatani, Y., Yamori, T., et al. (1999) Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors. *Oncogene* **18,** 813–822.

131. Sebolt-Leopold, J. S., Dudley, D. T., Herrera, R., et al. (1999) Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. *Nat. Med.* **5,** 810–816.

132. Lavoie, J. N., L'Allemain, G., Brunet, A., Muller, R., and Pouyssegur, J. (1996) Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. *J. Biol. Chem.* **271,** 20,608–20,616.

133. Woods, D., Parry, D., Cherwinski, H., Bosch, E., Lees, E., and McMahon, M. (1997) Raf-induced proliferation or cell cycle arrest is determined by the level of Raf activity with arrest mediated by p21Cip1. *Mol. Cell. Biol.* **17,** 5598–5611.

134. Treinies, I., Paterson, H. F., Hooper, S., Wilson, R., and Marshall, C. J. (1999) Activated MEK stimulates expression of AP-1 components independently of

phosphatidylinositol 3-kinase (PI3-kinase) but requires a PI3-kinase signal to stimulate DNA synthesis. *Mol. Cell. Biol.* **19,** 321–329.

135. Kawada, M., Yamagoe, S., Murakami, Y., Suzuki, K., Mizuno, S., and Uehara, Y. (1997) Induction of p27Kip1 degradation and anchorage independence by Ras through the MAP kinase signaling pathway. *Oncogene* **15,** 629–637.

136. Sewing, A., Wiseman, B., Lloyd, A. C., and Land, H. (1997) High-intensity Raf signal causes cell cycle arrest mediated by p21Cip1. *Mol. Cell. Biol.* **17,** 5588–5597.

137. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. (1997) Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* **88,** 593–602.

138. Lloyd, A. C., Obermuller, F., Staddon, S., Barth, C. F., McMahon, M., and Land, H. (1997) Cooperating oncogenes converge to regulate cyclin/cdk complexes. *Genes Dev.* **11,** 663–677.

139. Sidransky, D. and Hollstein, M. (1996) Clinical implications of the p53 gene. *Annu. Rev. Med.* **47,** 285–301.

140. Lin, A. W., Barradas, M., Stone, J. C., et al. (1998) Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. *Genes Dev.* **12,** 3008–3019.

141. Zhu, J., Woods, D., McMahon, M., and Bishop, J. M. (1998) Senescence of human fibroblasts induced by oncogenic Raf. *Genes Dev.* **12,** 2997–3007.

142. Wright, J. H., Munar, E., Jameson, D. R., et al. (1999) Mitogen-activated protein kinase kinase activity is required for the G(2)/M transition of the cell cycle in mammalian fibroblasts. *Proc. Natl. Acad. Sci. USA* **96,** 11,335–11,340.

143. Colanzi, A., Deerinck, T. J., Ellisman, M. H., and Malhotra, V. (2000) A specific activation of the mitogen-activated protein kinase kinase 1 (MEK1) is required for Golgi fragmentation during mitosis. *J. Cell. Biol.* **149,** 331–339.

144. Acharya, U., Mallabiabarrena, A., Acharya, J. K., and Malhotra, V. (1998) Signaling via mitogen-activated protein kinase kinase (MEK1) is required for Golgi fragmentation during mitosis. *Cell* **92,** 183–192.

145. Kharbanda, S., Saleem, A., Emoto, Y., Stone, R., Rapp, U., and Kufe, D. (1994) Activation of Raf-1 and mitogen-activated protein kinases during monocytic differentiation of human myeloid leukemia cells. *J. Biol. Chem.* **269,** 872–878.

146. Qiu, M. S. and Green, S. H. (1992) PC12 cell neuronal differentiation is associated with prolonged p21ras activity and consequent prolonged ERK activity. *Neuron* **9,** 705–717.

147. Alberola-Ila, J., Forbush, K. A., Seger, R., Krebs, E. G., and Perlmutter, R. M. (1995) Selective requirement for MAP kinase activation in thymocyte differentiation. *Nature* **373,** 620–623.

148. Tsai, M., Chen, R. H., Tam, S. Y., Blenis, J., and Galli, S. J. (1993) Activation of MAP kinases, pp90rsk and pp70-S6 kinases in mouse mast cells by signaling through the c-kit receptor tyrosine kinase or Fc epsilon RI: rapamycin inhibits activation of pp70-S6 kinase and proliferation in mouse mast cells. *Eur. J. Immunol.* **23,** 3286–3291.

149. Tsuda, L., Inoue, Y. H., Yoo, M. A., et al. (1993) protein kinase similar to MAP kinase activator acts downstream of the raf kinase in Drosophila. *Cell* **72,** 407–414.

150. Gabay, L., Seger, R., and Shilo, B. Z. (1997) In situ activation pattern of Drosophila EGF receptor pathway during development. *Science* **277,** 1103–1106.

151. Haccard, O., Sarcevic, B., Lewellyn, A., et al. (1993) Induction of metaphase arrest in cleaving Xenopus embryos by MAP kinase. *Science* **262,** 1262–1265.

152. Katsura, I. (1993) In search of new mutants in cell-signaling systems of the nematode *Caenorhabditis elegans*. *Genetica* **88,** 137–146.

153. Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J., and Greenberg, M. E. (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* **270,** 1326–1331.

154. von Gise, A., Lorenz, P., Wellbrock, C., et al. (2001) Apoptosis suppression by Raf-1 and MEK1 requires MEK- and phosphatidylinositol 3-kinase-dependent signals. *Mol. Cell. Biol.* **21,** 2324–2336.

155. Blagosklonny, M. V., Giannakakou, P., el-Deiry, W. S., Kingston, D. G., Higgs, P. I., Neckers, L., and Fojo, T. (1997) Raf-1/bcl-2 phosphorylation: a step from microtubule damage to cell death. *Cancer Res.* **57,** 130–135.

156. Blazquez, C., Galve-Roperh, I., and Guzman, M. (2000) De novo-synthesized ceramide signals apoptosis in astrocytes via extracellular signal-regulated kinase. *Faseb J.* **14,** 2315–2322.

157. Michael, D., Martin, K. C., Seger, R., Ning, M. M., Baston, R., and Kandel, E. R. (1998) Repeated pulses of serotonin required for long-term facilitation activate mitogen-activated protein kinase in sensory neurons of Aplysia. *Proc. Natl. Acad. Sci. USA* **95,** 1864–1869.

158. Berman, D. E., Hazvi, S., Rosenblum, K., Seger, R., and Dudai, Y. (1998) Specific and differential activation of mitogen-activated protein kinase cascades by unfamiliar taste in the insular cortex of the behaving rat. *J. Neurosci.* **18,** 10,037–10,044.

2

Determination of ERK Activity

Antiphospho-ERK Antibodies, In Vitro Phosphorylation, and In-Gel Kinase Assay

Sarah Kraus and Rony Seger

1. Introduction

The mitogen-activated protein kinases (MAPKs) are a family of protein serine/threonine kinases that operate within specific signaling pathways called MAPK cascades (for reviews see Chapter 1 and references therein). Each MAPK cascade is composed of up to six tiers of protein kinases, which activate each other, and thus participate in the amplification and specificity determination of the transmitted signals. Activation of the protein kinase components of the cascade is carried out by phosphorylation, which for enzymes at a given tier of the cascade occurs at a common phosphorylation site, such as the Thr-Xaa-Tyr motif for MAPKs. Eventually the signals are transmitted to several regulatory proteins that essentially govern all stimulated cellular processes including proliferation, differentiation, and response to stress.

Five distinct MAPK signaling cascades have been identified so far, and these are termed according to the components in the MAPK tier of the cascades. These cascades are (1) Extracellular signal-regulated kinase (ERK; *[1]*), (2) Jun N-terminal kinase (JNK; SAPK1 *[2,3]*), (3) p38MAPK (p38; SAPK2-4; *[4–6]*), and (4) Big MAPK (BMK, ERK5; *[7,8]*), also known as ERK5. A fifth kinase, ERK7, also contains the Thr-Xaa-Tyr motif and thus may represent a cascade that is not fully elucidated *(9)*. The different groups of MAPKs seem to differ in their physiologic activities since the ERKs usually play a role in proliferation and differentiation, whereas the other cascades seem to respond mainly to stress and to apoptotic stimuli. However, in the different tiers of each cascade, there

are two or more isoforms (e.g., ERK1, ERK1b, and ERK2; *[10]*) that under most circumstances execute similar physiologic functions.

The amount of signals transmitted via each MAPK cascade is important for understanding the outcome of studying intracellular signaling. Usually, the activity of one component of the MAPK level of each cascade (e.g., ERK, JNK, p38MAPK) is a sufficient indicator of the transmitted signal. However, for certain studies the activity of additional components within the cascades must be determined to understand the actual fate of the signal. For example, JNKs can be activated by several components in the MAPKK (MKK4 and 7; *[11,12]*) and MAP3K (e.g., MEKK1-4, ASK1 *[13]*), which seem to be held together by specific scaffold proteins *(14)*. Since such signaling complexes seem to operate simultaneously in response to certain stimuli, the study of several levels in the cascade is necessary to evaluate the amount of signal in the different branches of the JNK cascade that are formed by the different complexes.

Since most components of the MAPK cascades belong to the large family of protein kinases, singling out the activity of the studied protein kinase is essential. Several methods have been developed over the years to detect the activity of components of the MAPK cascades. One of the first methods used for the detection of protein kinases in growth factor signaling employed fractionation by MonoQ fast protein liquid chromatography *(15,16)*. This method involves examination of the resulting fractions of the MonoQ column for protein kinase activity. Since fractionation with the MonoQ column is extremely reproducible, kinases that are activated upon stimulation can be detected by comparing the elution profiles of kinases from activated and nonactivated cells. The fact that the protein kinases are eluted from the column allows determination of the actual kinase activity in solution rather than on any solid support. However, because the separation of various protein kinases is not always complete, and because its laborious nature, the method is not widely in use and is not described here.

Another method that is used for the detection of novel protein kinases is the in-gel kinase assay. This technique involves copolymerization of a given substrate in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the samples of interest on the copolymerized gel, and in-gel phosphorylation of the embedded substrate in the presence of $[\gamma^{32}P]$-ATP. The advantage of this method is that it reveals the molecular weight of the detected kinases, assisting in the identification of the enzymes of interest. The disadvantages of this procedure are the inability of certain protein kinases to renature, the length of the procedure, and the narrow linear range of the activities of the embedded kinases.

Since the MonoQ fractionation and in-gel kinase assay methods are lengthy and not always accurate, more specific and convenient methods are recom-

mended for the characterization of a given protein kinase. These methods often require specific reagents such as antibodies and affinity reagents for the isolation of the protein kinase of interest. Two important methods described here are detection of activity by antiphosphorylated MAPK antibodies *(17)* and by immunoprecipitation with specific antibodies followed by an in vitro kinase reaction *(18)*. However, detection of kinase activity based on a slower mobility of activated kinases on SDS-PAGE ("gel shift," "upshift") is not recommended because it does not always correlate with enzymatic activity. This was shown for ERK *(19)* and for Raf-1 *(20)*. Also note that although affinity techniques (including immunoprecipitation) are often used, the attachment to a solid support that occurs in these methods might interfere with the kinase activity. Thus, although these methods can give a good estimation regarding the relative activity of the kinase, they cannot be used when accurate kinetic data are required.

Several points have to be considered before attempting to determine the activity of any component in the MAPK cascades. One of the most important parameters to be considered is the method of protein extraction. The methods of choice should extract the protein kinases from the proper cellular compartment and, if necessary, preserve their active form while decreasing the amount of nonrelevant kinases. For example, activated Raf-1 can be present in mitochondrial membranes, which might not be disrupted by some extraction procedures, but are disrupted if RIPA buffer is used. Several methods have been developed for the proper extraction of MAPK components. Sonication, which disrupts the plasma membrane but does not solubilize it, is used to produce extracts that contain both cytosolic and some nuclear fraction. Solubilization by detergents (e.g., Triton X-100, NP-40) usually extracts proteins from the membrane and cytosol, although including SDS and deoxycholate among the detergents can extract proteins also from the nuclear compartment as well. Cellular extraction by addition of hot SDS-PAGE sample buffer is not recommended, because it frees chromatin, which is physically hard to handle. Extraction by freeze-thawing, is also not recommended, because of protein phosphatases that may act at low temperatures.

Another consideration is the inhibition of proteinases and/or protein phosphatases, which are released from cellular organelles on solubilization. Addition of specific inhibitors of phosphatases and proteinases to the extraction buffers and extraction at low temperatures minimize the effect of these enzymes. However, since phosphatases are usually efficient enzymes, extractions should be performed as fast as possible even if these precautions are taken. Furthermore, the quality of the antibodies employed is of great importance for the success of the various procedures below. These antibodies should recognize only the desired protein kinase, and not isoforms or nonrelevant enzymes. When in vitro kinase activity is determined, the antibodies should also not in-

terfere with the catalytic activity of the enzymes tested. Other parameters that should be considered for accurate comparison of protein kinase activity are amount of proteins for each assay, dilution and amount of antibodies, starvation of the cells before activation, optimal length of stimulation, and linear dynamic range of the phosphorylation reaction. Recommended amounts and concentrations are mentioned below; however, these should always be optimized for the particular cell line, stimuli, and MAPK component.

In this chapter, the main method for detecting MAPK signaling is the determination of MAPK activity by antiphospho antibodies. This method takes advantage of the fact that most MAPK components are activated by phosphorylation as already described. Thus, Western blot analysis with both antiphospho-MAPK antibody and general antibody would provide information on the specific and total activity of most MAPKs in a given fraction. This is an acceptable method, although it actually detects the phosphorylation by upstream components and dephosphorylation by phosphatases and therefore does not always reflect the actual activity of the tested kinase. Another assay involves immunoprecipitation and in vitro kinase assay. This method is quite convenient as well, although its disadvantage is that the kinase activity might be influenced by the solid support. Another method described herein is the "in-gel kinase assay," which is often used for the detection of novel kinases, but suffers from a limited linearity and lengthy procedures. An alternative method, using affinity reagents for the isolation of MAPK components as JNK, is described in other chapters.

2. Materials

All solutions should be prepared in distilled/deionized water.

2.1. Cell Culture and Protein Extraction

1. Dulbecco's modified Eagle's medium (DMEM) (#41965-039; Gibco-BRL).
2. Fetal calf serum (FCS) (#101-06078; Gibco-BRL), glutamine solution (Biological Industries, Beit Haemek, Israel), and antibiotics (Biolab, Jerusalem, Israel) stored in aliquots at –20°C.
3. Trypsin-EDTA (#T-3924; Sigma).
4. Stimulant: 50 µg/mL of epidermal growth factor (EGF) (#E-9644; Sigma) in EGF buffer (phosphate-buffered saline [PBS] containing 0.5 mg/mL of bovine serum albumin [BSA] [#A-9647; Sigma]).
5. 10X PBS, calcium and magnesium free (#14200-067; Gibco-BRL). Prepare 1X ice-cold PBS.
6. Homogenization buffer (buffer H) with protease inhibitors: 50 m*M* β-glycerophosphate (#G-6251; Sigma), pH 7.3, 1.5 m*M* EGTA, 1.0 m*M* EDTA, 1.0 m*M* dithiothreitol (DTT) (#D-9779; Sigma), 0.1 m*M* sodium orthovanadate, 1.0 m*M* benzamidine (#B-6506; Sigma), 10 µg/mL of aprotinin (#A-1153; Sigma), 10 µg/mL of leupeptin (#L-0649; Sigma), 2.0 µg/mL of pepstatin-A (#P-4265; Sigma).

7. Buffer A: 50 m*M* β-glycerophosphate, pH 7.3, 1.5 m*M* EGTA, 1.0 m*M* EDTA, 1.0 m*M* DTT, 0.1 m*M* sodium orthovanadate. Prepare 10X stock solution (without DTT) and store at –20°C. Prior to use add freshly prepared DTT.
8. Bradford reagent (Coomassie protein assay reagent, #BH44587; Pierce).

2.2. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

1. Gel electrophoresis apparatus and power supply.
2. 4X Laemmli reducing sample buffer: 0.2 *M* Tris-HCl, pH 6.8, 40% (v/v) glycerol; 8% (w/v) SDS, 8% (v/v) β-mercaptoethanol, 0.2% (w/v) bromophenol blue. Store aliquoted at –20°C.
3. Prestained molecular weight protein markers (#161-0305; Bio-Rad).
4. (30%) Acrylamide:(0.8%) bisacrylamide solution (#161-0158; Bio-Rad).
5. Lower (separating) buffer: 1.5 *M* Tris-HCl, pH 8.8.
6. Upper (stacking) buffer: 0.5 *M* Tris-HCl, pH 6.8.
7. Tetramethylethylenediamine (TEMED) (#161-0800; Bio-Rad).
8. 10% Ammonium persulfate (APS) (#161-0700; Bio-Rad).
9. Running buffer: 25 m*M* Tris, 192 m*M* glycine, 0.1% SDS, pH 8.3.
10. Staining solution: 40% methanol, 7% acetic acid, 0.005% bromophenol blue.
11. Destaining solution: 15% isopropanol, 7% acetic acid.

2.3. Western Blot Analysis

1. Transfer apparatus.
2. Transfer buffer: 15 m*M* Tris, 120 m*M* glycine, approximate pH of 8.8.
3. Nitrocellulose membrane (Protran BA 85; Schleicher & Schuell).
4. Whatman paper (3 mm).
5. Washing buffer (TBS-T): 20 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 0.05% Tween-20.
6. Blocking solution: 2% (w/v) BSA in washing buffer.
7. Primary antibody appropriate for signaling MAPK of interest; (e.g., monoclonal antidiphospho-ERK [M-8159] and polyclonal anti–general ERK [M-5670] from Sigma, Israel), and secondary antibody (alkaline phosphatase [AP]–or horseradish peroxidase [HRP]–conjugated antimouse or antirabbit Fab antibodies from Jackson) diluted in washing buffer to appropriate dilutions.
8. Enhanced chemiluminescence (ECL): Commercial kits are available from Amersham, Pierce, and Bio-Rad). Otherwise, ECL solutions can be made by mixing equal volumes of solution A (2.5 m*M* luminol [#A-8511; Sigma], 400 μ*M* p-coumaric acid [#C-9008; Sigma] in 100 m*M* Tris, pH 8.5) and solution B (5.4 m*M* H_2O_2 in 100 m*M* Tris-HCl, pH 8.5).
9. AP-based detection assay: nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) visualization solution comprised of 10 mL of AP substrate buffer (100 m*M* Tris-HCl, pH 9.5, 100 m*M* NaCl, 5.0 m*M* $MgCl_2$) containing 66 μL of NBT (50 mg/mL) (#S380C; Promega) and 33 μL of BCIP (50 mg/mL) (#S381C; Promega).

2.4. Immunoprecipitation

1. Antibodies for immunoprecipitation (e.g., anti-ERK C-terminus; C-16 Santa Cruz, CA).
2. Protein A-Sepharose (#P-3391; Sigma).
3. 0.5 *M* LiCl in 0.1 *M* Tris, pH 8.0.
4. Radioimmune precipitation (RIPA) buffer: 137 m*M* NaCl, 20 m*M* Tris-HCl, pH 7.4, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 0.5% (w/v) deoxycholate, 0.1% (w/v) SDS, 2.0 m*M* EDTA; 1.0 m*M* phenylmethylsulfonyl fluoride (#P-7626; Sigma); 20 μ*M* leupeptin.
5. Buffer A (*see* **Subheading 2.1., item 7**).
6. 3X Reaction mixture (RM) (with [γ^{32}P]-ATP): 75 m*M* β-glycerophosphate, pH 7.3; 100 μ*M* [γ^{32}P]-ATP (~4000 cpm/pmol) (Amersham or NEN); 0.3 m*M* unlabeled ATP, 30 m*M* MgCl$_2$, 2.5 mg/mL of BSA; 1.5 m*M* DTT; 3.75 m*M* EGTA; 0.15 m*M* sodium orthovanadate, 30 μ*M* calmidazolium (#288665; Calbiochem), 6 μ*M* PKI peptide (#116805; Calbiochem).
7. Substrate: 2 mg/mL of myelin basic protein (MBP) (bovine brain, #M-1891; Sigma).
8. Perspex shielding for radioactive work.

2.5. In-Gel Assay

1. Buffer H + 1% Triton X-100.
2. MBP (*see* **Subheading 2.4., item 7.**).
3. 20% Isopropanol, 50 m*M* HEPES, pH 7.6.
4. 6 *M* Urea in buffer A (*see* **Subheading 2.1., item 7.**).
5. Renaturation buffer: 50 m*M* HEPES, pH 7.6, containing 5.0 m*M* β-mercaptoethanol.
6. Renaturation buffer + 0.05% Tween-20.
7. In-gel kinase buffer: 20 m*M* HEPES, pH 7.6, containing 20 m*M* MgCl$_2.$
8. In-gel kinase/ATP buffer: in-gel kinase buffer containing 2.0 m*M* DTT, 20 μ*M* ATP, and 100 μCi of [γ^{32}P]-ATP.
9. 5% Trichloroacetic acid (TCA)–1% NaPPi.
10. Water bath at 30°C with proper shielding for radioactive work.

3. Methods

3.1. Cell Culture

Cultured cells (Rat1 or any other cell types, *see* **Note 1**) are maintained in growth medium (e.g., DMEM) supplemented with 10% heat-inactivated FCS, 1% glutamine, and an antibiotic mixture added to a final concentration of 100 U/mL of penicillin and 100 mg/mL of streptomycin. Heat inactivation of FCS is performed by heating it for 45 min at 56ºC. Cells are periodically harvested with trypsin-EDTA from confluent cultures. Prior to stimulation the cells are serum starved in starvation medium (DMEM containing 0.1% FCS) for 14–20 h. The

cells should not be removed from the incubator or handled in any other way at least 4 h before stimulation to avoid activation of the MAPKs owing to varying physical conditions (e.g., low temperature).

3.2. Preparation of Cell Extracts

One of the most important parameters for the successful determination of ERK activation is the proper extraction of the protein from the examined cell lines or tissues. We describe here an extraction by sonication, which is useful for cytosolic and nuclear proteins. However, other methods of extractions (e.g., by detergent) can be used as well, provided that inhibitors of phosphatases and proteinases are included in the extraction buffer at 4°C. The example used here for EGF stimulation of Rat1 cells can be employed with minor modifications for most cell types and stimuli.

1. Grow the cells (6-cm tissue culture plates) in DMEM containing 10% FCS to subconfluency (~0.5 × 10^6 cells/plate) in a tissue culture incubator (37°C, 5% CO$_2$).
2. Starve the cells (14–20 h) in starvation medium (2 mL/plate) (*see* **Note 2**).
3. Stimulate the cells by incubating them with 2 μL of EGF (final concentration can vary between 5 and 100 μ*M*) for various time points. Control plates should be treated with EGF buffer alone for the same times as for the EGF treatment (*see* **Notes 3** and **4**).
4. At the appropriate time interval, remove the medium from the plates. Then, rinse the plates twice with ice-cold PBS and once with ice-cold buffer A (5 mL each). Since the arrest and slowing down of biologic processes is desired at this stage, it is recommended that the plates be placed on ice.
5. Add 200 μL of ice-cold buffer H to each plate, tilt the plate gently and scrape the cells using a plastic scraper. Transfer the cells to labeled, precooled 1.5-mL plastic Eppendorf tubes (*see* **Note 5**).
6. Disrupt the cells by sonication (two 7-s 50-W pulses) on ice.
7. Centrifuge the cellular extracts at 14,000*g* for 15 min at 4°C. The supernatant contains the cytosolic extracts to be examined for phosphorylation (*see* **Note 6**); transfer to new, precooled test tubes.
8. Take aliquots (5–10 μL) from the resulting supernatants for protein determination. Store the remainder of each cytosolic extract on ice until needed.
9. Dilute the samples (usually 1:20) to make sure that the protein concentration is within the dynamic range of the detection (within the concentration of the used standards) and proceed as follows:
   a. Put 10 μL of each protein standard (5, 10, 20, 50, 100. and 200 μg/mL of BSA in buffer H) into at least two wells of a flat-bottomed 96-well microplate.
   b. Put 10 μL of each of the diluted samples in duplicates. Add 190 μL of Bradford reagent to all the wells.
   c. Place the microplate in a microplate reader and determine the optical density (OD) of the samples at 595 n*M*. From the ODs, calculate the protein concentrations of the samples.

10. Equal amounts of cell extract from each of the treatments (*see* **step 3**) are used for Western blotting (usually 20 μg of protein/sample), immunoprecipitation (usually 300 μg), or in-gel kinase assay (100 μg). For Western blot analysis, add to each of the samples 1/3 vol of 4X sample buffer, mix the contents, boil for 3 min, and spin for 1 min at 14,000*g*. For immunoprecipitation, incubate the cytosolic extracts with the antibodies as described below. For in-gel assay, mix the cytosolic extracts with 1/3 vol of 4X sample buffer without boiling and separate the proteins on proper gels as described below.

3.3. Western Blot Analysis and Antibodies

1. For Western blot analysis, proteins are first separated by 10 or 12% SDS-PAGE. To prepare the gel, first assemble glass plates and spacers in a minigel apparatus (Bio-Rad). Prepare 10% polyacrylamide separating gel (10 mL) by mixing 3.3 mL of acrylamide stock solution, 2.5 mL of lower buffer, 4.2 mL of water, 100 μL of APS, and 10 μL of TEMED. Insert ~7.5 mL into the glass plates. Overlay the separating gel with water and allow gel to polymerize.

2. Prepare 5 mL of 3% polyacrylamide stacking gel by mixing 750 μL of acrylamide stock solution, 1.25 mL of upper buffer, 3.0 mL of water, 100 μL of APS, and 10 μL of TEMED. Cast the gel, insert a comb, and allow to polymerize. Assemble the gel in the apparatus and add running buffer.

3. Load the prepared samples above and a prestained protein marker on the gel and run the gel at 150 V. Once the dye front of the SDS-PAGE has reached the end of the gel, remove the gel from the apparatus, and proceed with the transfer step (*see* **Note 7**).

4. Prewet (soak) the nitrocellulose membrane in transfer buffer.

5. Fill the transfer apparatus with transfer buffer. Make a sandwich of the SDS-gel, nitrocellulose membrane, and transfer pads by placing a wet (transfer buffer) 3-mm piece of Whatman paper on a wet pad, the gel on top of the Whatman paper, the wet nitrocellulose membrane on top of the gel, and the other wet 3-mm Whatman paper on top of the nitrocellulose membrane.

6. Remove any air bubbles from between the different layers of the transfer sandwich by gently rolling a 10-mL pipet over the sandwich. Place the other wet pad on top of the transfer sandwich. Make sure air bubbles are not trapped between the gel and the other components.

7. Place the sandwich containing the SDS-gel and nitrocellulose membrane into the buffer-filled transfer apparatus. The nitrocellulose membrane should face the side with the cathode and the SDS-gel should face the side with the anode. Connect the apparatus to a power supply and start the current (200-mA constant current, 90 min, preferably with a cooling device). Methanol or 0.05% SDS are sometimes included in the transfer buffer; their inclusion will require different transfer conditions.

8. At the end of the transfer period, turn off the power supply and remove the nitrocellulose membrane from the transfer sandwich. Rinse the nitrocellulose membrane with transfer buffer to remove any adhering pieces of gel and place the membrane in a flat container.

9. Incubate the nitrocellulose membrane in blocking solution for 60 min at room temperature (*see* **Note 8**).
10. Incubate the blot with the first antibody (monoclonal anti–active ERK antibody, diluted according to the manufacturer's recommendations). This incubation can be either overnight at 4°C, 30 min at 37°C, or 1 to 2 h at room temperature(*see* **Note 9**).
11. Wash the blot in the flat container at least three times for 15 min each with TBS-T at 23°C.
12. Incubate the blot with the second antibody (AP/ECL-conjugated goat antimouse IgG diluted according to the manufacturer' instructions in TBS-T) for 45 min at room temperature.
13. Wash the blot at least three times for 10 min each with TBS-T.
14. Use an AP/ECL detection protocol to detect phosphorylated ERK (*see* **Note 10**).
15. After detecting the phosphorylated ERK, it is recommended that one determine whether there is an equal amount of ERK using antigeneral ERK antibody. Note that the different antibodies may interfere with the detection of each other, and, therefore, either additional identical blot or a stripping step is required. For the second staining of the same nitrocellulose, incubate it in blocking solution for 30 min at room temperature.
16. Incubate the blot with the "new" first antibody (polyclonal anti-general ERK antibody). Develop as above with the HRP/AP system that had not been used for the first step and the appropriate ECL/AP system. Two or three bands are usually stained by the antibodies. When two bands appear, these are the p42 ERK2 and p44 ERK1. In some cell lines and tissues, a third band at 46 kDa is detected (ERK1b). The intensity of staining of the bands is elevated and this reflects their time course of regulatory phosphorylation upon stimulation (**Fig. 1**), while the amount of the ERK as detected by the anti-total ERK antibody is not changed for up to 2 h of stimulation (**Fig. 1**).

3.4. Determination of ERK Activity by Immunoprecipitation

Determining ERK activity by immunoprecipitation involves the isolation of the enzyme using immunoprecipitation with specific antibodies and then performing a phosphorylation reaction in vitro. Although ERK is used here as an example, if appropriate reagents are used, this protocol can be performed with most MAPK isoforms and other components of the MAPK cascade. This protocol facilitates a fast and efficient isolation of the kinase of interest and its reliable quantitation by a phosphorylation reaction. For immunoprecipitation, specific antibodies directed to the C-terminal domain of the ERK are used. The quality and specificity of the antibodies used for the immunoprecipitation protocol is particularly important. Usually, anti-C-terminal ERK antibodies are used, which do not interfere with the enzymatic activity of the kinase tested.

In this assay the amount of proteins in the different samples and the dilution of antibodies should be optimized to avoid nonspecific recognition of excess
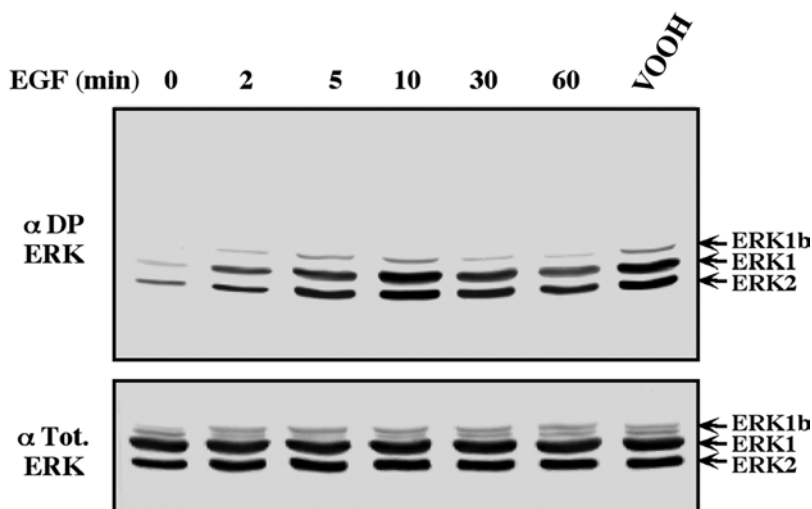
Fig. 1. Detection of ERK activity by Western blotting with antidiphospho ERK antibody. Subconfluent Rat1 cells were serum starved (DMEM + 0.1% FCS, 18 h) and then treated with either EGF (50 ng/mL) for the indicated times, VOOH (100 μM sodium orthovanadate and 200 μM H_2O_2) for 20 min, or left untreated (basal control). Cytosolic extracts were prepared as described. Samples (20 μg) were prepared, separated by a 10% SDS-PAGE, and blotted with either the antidiphospho ERK antibody **(top)** or with anti-total ERK antibody **(bottom)**. This was followed by development with the AP system. The site of ERK2, ERK1 and ERK1b is indicated.

proteins. The stringent washings of the immunoprecipitates are necessary to avoid nonspecific precipitation of contaminant kinases. In addition, this assay is performed while the enzyme is still on the beads, and, therefore, the results obtained do not accurately reflect the specific activity of ERK (qualitative and not quantitative). For accurate kinetic data, it is possible to elute the protein kinase from the immunoprecipitating beads (or isolate them by other means) and then determine their activity in solution (*see* **Notes 11** and **12**). The protocol is as follows:

1. As above, the assay is described for six samples. The protein A-Sepharose beads described are supplied as a dry powder; in case the beads are preswollen, proceed from **step 4**.
2. Place the protein A-Sepharose beads (~150 μL) in a 1.5-mL plastic test tube, add 1 mL of PBS, and let the beads swell for 10 min at room temperature.
3. Wash the swollen beads three times with 1 mL of PBS (resuspend in buffer and centrifuge for 1 min at 14,000*g*, at room temperature. Discard the supernatant.
4. Add 15 μL of the antibodies to be conjugated to 120 μL of the swollen packed beads and 365 μL of PBS (final volume of 0.5 mL). Rotate the mixture (1 h at

room temperature) on an end-by-end rotator to allow the antibodies to bind to the protein A (this can be done at 4°C, 16 h). The volumes listed here should be sufficient for eight reactions but, because of the density of the beads, will probably only be sufficient for six or seven reactions.

5. Wash the beads once with 1 mL of ice-cold PBS and then three times with 1 ml ice-cold buffer H (all at 4°C). Resuspend the washed beads in an equal volume of ice-cold buffer H (~250 μL for ~250 μL of beads). Either use the antibody-conjugated beads immediately, or store at 4°C until used. It is best to use the conjugated beads within 3 d of preparation.

6. Add 30 μL of the antibody-conjugated bead suspension (15 μL net) to a 300-μL sample of cytosolic extract containing 50–500 μg of total protein (in buffer H) in precooled 1.5-mL plastic test tubes. Rotate end to end for 2 h at 4°C. Although this is not always necessary, we recommend using equal amounts of protein in each of the samples to be immunoprecipitated to avoid inaccuracy.

7. Centrifuge the incubation mixture for 1 min at 14,000*g* and 4°C. Remove and discard the incubation supernatant from the antibody-conjugated beads. Wash the beads once with 1 mL of ice-cold RIPA buffer, twice with ice-cold 0.5 *M* LiCl, and twice with 1 mL of ice-cold buffer A. As previously mentioned, these stringent washes are important, because they remove "sticky" protein kinases that might interact nonspecifically with the protein A beads.

8. After the last washing step, remove buffer A completely from the conjugated beads and resuspend the pellets of the beads in 15 μL of double-distilled water.

9. At this stage, prepare your work bench for working with a small amount of radioactivity and add 10 μL of 3X RM to each tube (*see* **Note 13**).

10. Start the phosphorylation reaction by adding 5 μL of the phosphorylation substrate (MBP, 2 mg/mL), or other substrate to the tube and placing the mixture in a thermomixer at 30ºC (*see* **Note 14**).

11. Incubate for 10–20 min at 30°C with either constant or frequent shaking. If a thermomixer is not available, a water bath or other heating device can be used.

12. End the phosphorylation reactions by adding 10 μL of 4X sample buffer to each tube. Boil, centrifuge (1 min at 14,000*g*), and load the supernatants on a 15% SDS-PAGE gel.

13. When the front dye of the gel reaches about 0.5 cm from the bottom of the gel, stop the current. To remove the excess free radiolabeled ATP, which migrates just in front of the bromophenol blue, cut out the part of the gel below the dye. This will considerably reduce the amount of radioactivity in the gel.

14. Transfer the separated proteins onto a nitrocellulose paper using a blotting apparatus as described in **Subheading 3.3.**. Wash briefly with distilled water and let dry. An alternative way would be to stain, destain, and dry the gel on a Whatman 3-mm paper, but this procedure does not allow further detection of proteins in the gel as described for the immunoprecipitated ERKs in **step 16**.

15. Expose the gel in a phosphorimager or on X-ray film (at –80°C). A band should appear at 16–21 kDa, which is the molecular weight of the four MBP isoforms.

16. To make sure that an equal amount of ERK was immunoprecipitated in each treatment, the nitrocellulose can then be blocked with BSA, overlayed with anti–
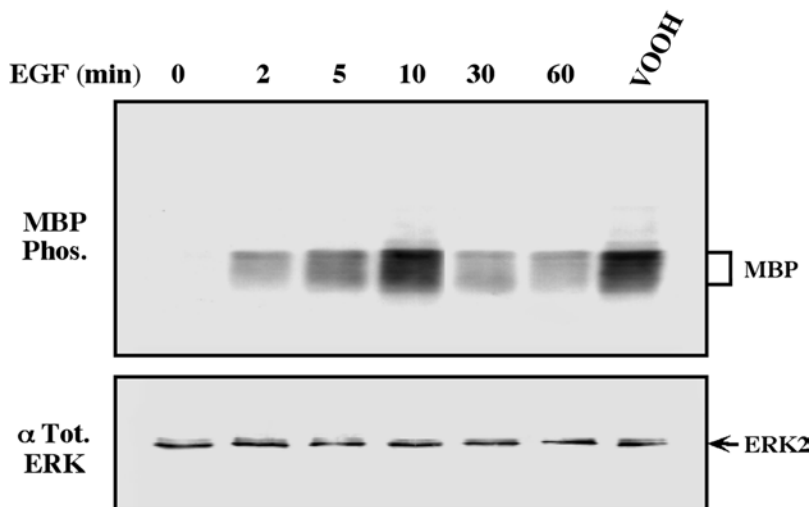
Fig. 2. Detection of ERK activity using in vitro kinase assay. Subconfluent Rat1 cells were serum starved (DMEM + 0.1% FCS, 18 h) and then treated with either EGF (50 ng/mL) for the indicated times, VOOH (100 μM sodium orthovanadate and 200 μM H_2O_2) for 20 min, or left untreated (basal control). Cytosolic extracts were prepared as described. For immunoprecipitation and in vitro kinase assay, proteins (300 μg) were incubated with 30 μL of anti-ERK C-terminus antibody-conjugated protein A beads. Phosphorylation reaction on MBP was performed as described and terminated by boiling in sample buffer. The proteins were then separated by 15% SDS-PAGE, blotted onto nitrocellulose, and subjected to autoradiography **(top)**, and to anti-total ERK antibody **(bottom)**. The sites of MBP and of ERK2 are indicated.

general ERK antibodies and developed (*see* **Subheading 3.3.**). Special precaution should be taken because of the radioactivity (*see* **Note 15**).

17. Following exposure on an X-ray film, phosphorylation is detected on a group of bands at 12–21 kDa, which are the different isoforms of MBP (**Fig. 2**). The intensity of phosphorylation in each time is changed and reflects the time course of activation of the ERKs. When the amount of ERKs is detected by the anti-total ERK antibody, primarily ERK2 can be detected at 42 kDa (**Fig. 2**)

3.5. In-Gel Kinase Assay

If the identity of the kinase is not known, or there are no specific antibodies available for the kinase, the in-gel kinase assay may be used instead of the basic immunoprecipitation method. This in-gel protocol involves copolymerizing a substrate with SDS and polyacrylamide and electrophoresis of the protein sample on the resulting SDS-polyacrylamide gel. After several rounds of denaturation and renaturation, a phosphorylation reaction is performed on the

gel and the phosphorylated bands are visualized by X-ray film or by a phosphorimager. With this method, the molecular weight of the protein kinase is revealed, and novel protein kinases can be identified. However, not all protein kinases can be renatured under the conditions of this protocol, and the linear range of this assay is usually limited. Therefore, this method should not be routinely used to monitor and characterize known protein kinases. The protocol is as follows:

1. To prepare the samples, follow the same protocol as described in **Subheading 3.2., steps 1–3**.
2. Harvest cells in buffer H + 1% Triton X-100 on ice, and centrifuge (14,000g, 15 min, 4°C), and determine the protein concentration of the samples (*see* **Subheading 3.2., steps 8** and **9**).
3. Add 1/4 vol of 4X sample buffer to 30–80 µg of extract. *Do not boil*. Keep at 4°C.
4. Prepare a 12% SDS-polyacrylamide separating gel containing MBP (0.5 mg/mL) (*see* **Notes 17** and **18**). This is done by mixing 4 mL of acrylamide/bisacrylamide solution, 2.5 mL of lower (separating) buffer, 2.5 mL of MBP (2 mg/mL), and 0.9 mL of water. Add 100 µL of APS and 10 µL of TEMED and cast in the separating aparatus as described above. Prepare the lower gel with 7.5 mL of the above solution in a minigel aparatus (Bio-Rad) and let polymerize. Prepare the upper stacking gel without MBP as described in **Subheading 3.3., step 2**. Load the samples and start the electrophoresis; in order to avoid heating, do not exceed 70–100 V.
5. Place the gel (without the stacking gel) in a flat container and wash it twice (30 min each at room temperature) with 100 mL of 20% isopropanol-50 mM HEPES (pH 7.6), twice (30 min each at room temperature) with renaturation buffer, and twice (15 min each at room temperature) with 100 mL of 6 M urea. If necessary, the second wash with 20% isopropanol-50 mM HEPES (pH 7.6) can be done overnight at 4°C.
6. Place the gel in a cold room (4°C), remove 50 mL of the 6 M urea, add 50 mL of renaturation buffer + 0.05% Tween-20, and shake for 15 min. (The washing solution is now 3 M urea in renaturation buffer + Tween-20.) Remove again 50 mL of the washing solution, add 50 mL of renaturation buffer + 0.05% Tween-20, and shake for an additional 15 min (reduce the urea to 1.5 M). Repeat once more so that the washing solution will be 0.75 M urea in renaturation buffer + Tween-20. Then, wash the gel three times, 15 min each with 100 mL of renaturation buffer + 0.05% Tween-20. Shake the gel overnight in the cold room.
7. Remove the washing buffer and incubate the gel in 30 mL of in-gel kinase buffer at 30°C for 30 min. Remove the buffer; add 20 mL of in-gel kinase buffer containing 2 mM DTT, 20 µM ATP and 100 µCi of [γ^{32} P]-ATP; and incubate at 30°C for 2 h. At this stage, the amount of radioactive material is very high, and, therefore, the reaction should be performed with a proper shielding. Make sure that the gel is straight in the flat container. Unequal distribution of the phosphorylation buffer may lead to wrong concentration of the ingredients and interfere with the phosphorylation reaction.
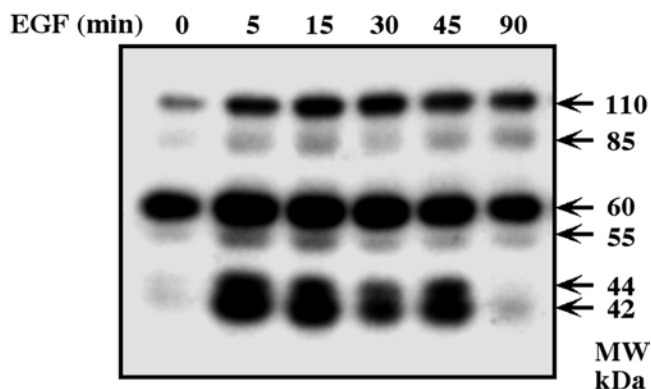
Fig. 3. Detection of MAPK activities by in-gel kinase assay. Subconfluent CHO cells overexpressing the ErbB-2 receptor were serum starved (DMEM + 0.1% FCS, 18 h) and then treated with EGF (50 ng/mL) for the indicated times. In-gel kinase assay was performed as described in **Subheading 3.5.** Arrows indicate the calculated molecular weight of the bands.

8. Wash the gel carefully four times (15 min each at room temperature) with 5% TCA + 1% NaPPi. If the gel is still very radioactive, continue washing overnight.
9. Dry the gel and subject to autoradiography. Bands should appear where kinases are present and caused phosphorylation of the MBP copolymerized in the gel.
10. The rate of phosphorylation of up to nine protein kinases can be enhanced by different treatment, although not all of them can be seen under all conditions. In **Fig. 3**, phosphorylation by six protein kinases is enhanced in Chinese hamster ovary (CHO) cells overexpressing ErbB2 on stimulation with EGF. These are the three bands at 4244 kDa (ERK2, ERK1), and bands with unknown identity at 55, 60, 85, and 110 kDa.

4. Notes

1. The methodology is suitable for a wide range of cell types and agonists. The methods described here were originally developed for cells grown in monolayer cultures. It may be necessary to adapt them for other (nonadherent) cell types. Although this protocol describes EGF stimulation of cells, this procedure, with minor changes, can be used for most extracellularly stimulated cells.
2. One of the parameters that should be considered before activation of cells by any stimulus is serum starvation (serum deprivation), which is usually done in 0.1% serum or sometimes even without serum at all. The aim of this starvation, which makes the cells quiescent, is to significantly reduce the amount of inducible MAPK phosphatases and to obtain a lower basal activity and thus extend the possible fold activation. For most cells, this can be achieved within 14 h. Starva-

tion for too long, or any change in temperature or pH, may be stressful to the cells, and thereby induce activation of one or more signaling pathways.

3. The optimal length of stimulation may vary among stimuli, cells, and other conditions. Thus, appropriate time courses for each kinase should be determined to obtain an accurate stimulation by various stimuli.

4. Positive and negative controls are very important for the success of the experiments. Negative control is a plate that was not exposed to any stimulant or to the vehicle used to dissolve the stimulant. Because of its importance as a baseline for the whole experiment, we use as negative controls either two plates or one plate for each time point and concentration. If the influence of the stimulating agent on the cells is not yet known it is recommended that a positive control be included in each experiment, such as peroxovanadate (VOOH), which nonspecifically activates many signaling events *(21)*.

5. Special consideration should be given to the composition of buffer H *(22)*. It is recommended that β-glycerophosphate be used, which serves both as a buffer and as a general phosphatase inhibitor, rather than Tris or HEPES. Sodium orthovanadate is used to inhibit tyrosine phosphatases and the mixture of pepstatin-A, aprotinin, leupeptin, and benzamidine are used to inhibit proteases. This buffer, when cold, blocks most of the phosphatase and proteinase activities in cell extracts. Addition of specific inhibitors of phosphatases and proteases and extraction at low temperatures minimize the effect of these enzymes. However, since phosphatases are usually efficient enzymes, even if these precautions are taken, extractions should be performed as quickly as possible.

6. As mentioned, the method of protein extraction is an important parameter in the determination of activity of any cellular enzyme. Since MAPKs are localized within cells, the cellular membranes must be disrupted to access the desired targets. The protein kinases of interest must then be obtained and preserved in their active form, while decreasing the amount of nonrelevant kinases. For more details *see* **Subheading 1.**

7. In the Western blot step, the efficiency of protein transfer is usually monitored visually by the transfer of prestained protein markers from the gel to the nitrocellulose membrane. The total amount of protein transferred can also be detected by staining the nitrocellulose membrane with Ponceau red. However, since the total amount of nonphosphorylated protein is determined by general antibodies as described, staining with Ponceau red is probably not essential for this particular protocol.

8. For blocking of the nitrocellulose membrane, we usually use BSA. Although BSA is considered relatively expensive, it is often used as a blocking solution in the Western blot procedure. The use of nonfat dry milk is not always recommended, because it can cause high background owing to phosphotyrosine-containing proteins in the milk, or it may contain phosphatases.

9. The successful use of sequence-specific antiphosphoprotein antibodies relies on their specificity for the phosphorylated form of the examined protein (see anti-MAPK antibodies below). Monoclonal antibodies, which usually confer better specificity than polyclonal ones, are considered a reliable tool for distinguishing

phosphorylated from nonphosphorylated forms of proteins, although affinity-purified polyclonal antibodies can be used as well.

10. For accurate comparison of the amounts of phosphoproteins, detection should be performed in the linear range of the detection system. Thus, the amount of protein loaded on the gel, the concentration of primary and secondary antibodies, and the time of ECL exposure should be optimized in order to reach linearity. Alternatively, a standard curve with the proteins of interest can be made and serial dilutions of the cellular extracts of each treatment can be loaded to the SDS-PAGE. The blotting detection systems, such as ECL-, [125]I-, AP-, or biotin-conjugated antibodies, should be chosen carefully. Usually, ECL has the narrowest linear range of these systems, whereas ^{125}I antibodies have a relatively broad range. The AP detection system, which has a moderate linear range, is usually used for the types of experiments described here, because it is a convenient method.

11. Immunoprecipitation methods vary in the order in which the antibodies and protein A are added to the cell extracts. In the protocol described here, the antibodies are conjugated to protein A beads, and only then added to the cytosolic extracts. This procedure minimizes the time of incubation of the samples with the antibodies, and thereby minimizes exposure of the desired kinases to phosphatases and proteinases in the extracts. Furthermore, this procedure ensures that only antibodies recognized by protein A will be used for the immunoprecipitation. In this case, antibodies that are not recognized by protein A are able to bind the desired antigen but then cannot be precipitated when protein A beads are added, and, therefore, the efficiency of immunoprecipitation is reduced.

12. The antibodies used for immunoprecipitation should not mask the kinase activity of the MAPKs. Specific antibody directed to the C-terminus of ERK is usually used for this purpose. If the nature of the antibody is not known, it is recommended that nonrelated antibody be used in parallel with the examined antibody as a control for the efficiency of the immunoprecipitation.

13. In the in vitro phosphorylation step, the composition of the reaction mixture (3X RM) is important for optimal ERK activity. The most important components of the reaction mixture are the Mg^{+2} and $[\gamma^{32}P]$-ATP, which are essential for the phosphorylation reaction. We recommend the use of 100 μM ATP with ~4000 cpm/pmol of the labeled ATP, which provides an extended linear range and reproducible results. When the enzymatic activity of the kinases is very low, which makes detection of phosphorylation difficult, the concentration of cold ATP should be reduced to 10–20 μM and the amount of radioactive material elevated. Addition of labeled ATP alone is not recommended because this will result in a nanomolar concentration of ATP, which is much below the *Km* for ATP and may lead to nonspecific phosphorylation. As previously mentioned, the β-glycerophosphate in the reaction mixture serves as a buffer but can also inhibit residual phosphatases that may have nonspecifically bound to the beads. The BSA serves as a carrier protein but when purity is required, it can be eliminated. The EGTA chelates Ca^{2+}, which may interfere with some kinase activities; DTT

keeps the proteins reduced; and sodium orthovanadate inhibits tyrosine phosphatases. Additional protein kinase inhibitors may be included in the mixture such as calmidazolium (calmodulin antagonist) and PKI peptide (protein kinase A inhibitor).

14. Substrates used in the phosphorylation reaction should be well phosphorylated by the desired kinases to allow accurate detection of the phosphorylation reaction. MBP can serve as a good, nonspecific substrate for many protein kinases including ERKs, although it is probably not a physiologic substrate for any MAPK. However, more specific substrates are often used and these include the purified, recombinant RSK, MNK, or Elk1 and peptides made according to the phosphorylation sites on this protein.

15. As mentioned, the determination of enzymatic activity when enzymes (in this case MAPKs) are bound to beads is not always accurate. One solution for this problem is to release the kinase(s) of interest from the beads, by adding excess immunizing peptide. The phosphorylation reaction can then be performed without interference of the beads, and the activity can be measured by a "paper assay" *(22)*.

16. As mentioned for the Western blot technique, for accurate comparison of the activities of protein kinases, detection should be performed in the linear range of the phosphorylation reaction. Thus, the amount of protein used for immunoprecipitation, the concentration of antibodies, the length of the phosphorylation reaction and the exposure to X-ray film or to the phosphorimager should be optimized in order to reach linearity. If necessary, a standard curve with the protein kinases of interest can be made, and serial dilutions of the cytosolic extracts or a time course of the phosphorylation can be used to ensure that one is working in a linear range.

17. MBP serves as an extremely good substrate for the in-gel kinase assay, but other substrates can be used as well. The concentration of the substrate is very important for strong signals, and a concentration of 0.2–0.6 mg/mL of the embedded proteins is recommended. It is important to make sure that the gel is not heated above 30°C; therefore, the voltage used for electrophoresis should be not higher than 100 V. Not all protein kinases can be denatured on the recommended procedure, and other methods of denaturation-denaturation such as the use of guanidium HCl can sometimes be used with slightly different results.

18. It is recommended that proper positive and negative controls be used for the in-gel assay. In particular, a gel without any embedded substrate should be used to verify that the phosphate incorporation is owing to phosphorylation of the exogenous substrate.

Acknowledgments

References

 1. Boulton, T. G., Yancopoulos, G. D., Gregory, J. S., Slaughter, C., Moomaw, C. J. H., and Cobb, M. H. (1990) An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. *Science* **249,** 64–67.
 2. Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993) Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes Dev.* **7,** 2135–2148.
 3. Kyriakis, J. M., Banerjee, P., Nikolakaki, E., et al. (1994) The stress-activated protein kinase subfamily of c-Jun kinases. *Nature* **369,** 156–160.
 4. Freshney, N. W., Rawlinson, L., Guesdon, F., et al. (1994) Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of Hsp27. *Cell* **78,** 1039–1049.
 5. Han, J., Lee, J. D., Bibbs, L., and Ulevitch, R. J. (1994) A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* **265,** 808–811.
 6. Rouse, J., Cohen, P., Trigon, S., et al. (1994) A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell* **78,** 1027–1037.
 7. Lee, J. D., Ulevitch, R. J., and Han, J. (1995) Primary structure of BMK1: a new mammalian map kinase. *Biochem. Biophys. Res. Commun.* **213,** 715–724.
 8. Zhou, G., Bao, Z. Q., and Dixon, J. E. (1995) Components of a new human protein kinase signal transduction pathway. *J. Biol. Chem.* **270,** 12,665–12,669.
 9. Abe, M. K., Kuo, W. L., Hershenson, M. B., and Rosner, M. R. (1999) Extracellular signal-regulated kinase 7 (ERK7), a novel ERK with a C-terminal domain that regulates its activity, its cellular localization, and cell growth. *Mol. Cell. Biol.* **19,** 1301–1312.
10. Yung, Y., Yao, Z., Hanoch, T., and Seger, R. (2000) ERK1b: a 46 kD ERK isoform that is differentially regulated by MEK. *J. Biol. Chem.* **275,** 15,799–15,808.
11. Derijard, B., Raingeaud, J., Barrett, T., et al. (1995) Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms [published erratum appears in *Science* 1995 Jul 7;269(5220):17]. *Science* **267,** 682–685.
12. Tournier, C., Whitmarsh, A. J., Cavanagh, J., Barrett, T., and Davis, R. J. (1997) Mitogen-activated protein kinase kinase 7 is an activator of the c-Jun NH2-terminal kinase. *Proc. Natl. Acad. Sci. USA* **94,** 7337–7342.
13. Kyriakis, J. M. (1999) Making the connection: coupling of stress-activated ERK/MAPK (extracellular-signal-regulated kinase/mitogen-activated protein kinase) core signalling modules to extracellular stimuli and biological responses. *Biochem. Soc. Symp.* **64,** 29–48.
14. Pawson, T. and Scott, J. D. (1997) Signaling through scaffold, anchoring, and adaptor proteins. *Science* **278,** 2075–2080.
15. Ahn, N. G. and Krebs, E. G. (1990) Evidence for an epidermal growth factor-stimulated protein kinase cascade in Swiss 3T3 cells. Activation of serine peptide kinase activity by myelin basic protein kinases in vitro. *J. Biol. Chem.* **265,** 11,495–11,501.

16. Ahn, N. G., Seger, R., Bratlien, R. L., Diltz, C. D., Tonks, N. K., and Krebs, E. G. (1991) Multiple components in an epidermal growth factor-stimulated protein kinase cascade. *In vitro* activation of myelin basic protein/microtubule-associated protein-2 kinase. *J. Biol. Chem.* **266,** 4220–4227.
17. Yung, Y., Dolginov, Y., Yao, Z., et al. (1997) Detection of ERK activation by a novel monoclonal antibody. *FEBS Lett.* **408,** 292–296.
18. Jaaro, H., Rubinfeld, H., Hanoch, T., and Seger, R. (1997) Nuclear translocation of mitogen-activated protein kinase kinase (MEK1) in response to mitogenic stimulation. *Proc. Natl. Acad. Sci. USA* **94,** 3742–3747.
19. Yao, Z., Dolginov, Y., Hanoch, T., et al. (2000) Detection of partially phosphorylated forms of ERK by monoclonal antibodies reveals spatial regulation of ERK activity by phosphatases. *FEBS Lett* **468,** 37–42.
20. Force, T., Bonventre, J. V., Heidecker, G., Rapp, U., Avruch, J., and Kyriakis, J. M. (1994) Enzymatic characteristics of the c-Raf-1 protein kinase. *Proc. Natl. Acad. Sci. USA* **91,** 1270–1274.
21. Zhao, Z., Tan, Z., Diltz, C. D., You, M., and Fischer, E. H. (1996) Activation of mitogen-activated protein (MAP) kinase pathway by pervanadate, a potent inhibitor of tyrosine phosphatases. *J. Biol. Chem.* **271,** 22,251–22,255.
22. Ahn, N. G., Weiel, J. E., Chan, C. P., and Krebs, E. G. (1990) Identification of multiple epidermal growth factor-stimulated protein serine/threonine kinases from Swiss 3T3 cells. *J. Biol. Chem.* **265,** 11,487–11,494.

3

Detection of ERK1/2 Activities Using Affinity Reagents

Rafael Pulido, Ángel Zúñiga, and Axel Ullrich

1. Introduction

The three major mammalian mitogen-activated protein kinase (MAPK) sub-families include the extracellular signal-regulated kinases (ERK1/2), the c-Jun N-terminal kinases (JNKs), and the p38 kinases. These kinases are differentially activated in response to extracellular stimuli and show differential specificity toward their substrates, which is provided, in part, by the existence on the different substrates of specific kinase docking sites (reviewed in **ref. *1***). Specific substrates of JNKs and p38s include the transcription factors c-Jun and ATF2, respectively, and GST-c-Jun and GST-ATF2 fusion proteins are widely used as suitable substrates to measure in vitro the kinase activity of these enzymes *(2–5)*. On the other hand, although a wide number of cytosolic, membrane-bound, and nuclear proteins are substrates of ERK1/2 (reviewed in **refs. *6*** and ***7***), measurement of ERK1/2 kinase activity in vitro is routinely performed using the nonphysiologic substrate myelin basic protein (MBP) *(8,9)*. ERK1/2 and p38α bind to the protein tyrosine phosphatase PTP-SL through a kinase interaction motif (KIM) located outside of the protein tyrosine phosphatase (PTP) catalytic domain; on binding, PTP-SL is phosphorylated by these MAPKs, mainly at the Thr^{253} residue *(10,11)*. Phosphorylation by active ERK1/2 and p38α of a GST-PTP-SL fusion protein containing the KIM and the MAPKs phosphorylation site (GST-PTP-SL 147-288) is illustrated in **Fig. 1**, in comparison with the phosphorylation of MBP. As shown, both GST-PTP-SL 147-288 and MBP are phosphorylated at a comparable extent by active MAP kinases (either endogenous [**Fig. 1A**] or recombinant [**Fig. 1B**]) in immune complex in vitro kinase assays. On the other hand, the in vitro activity of ERK1/2 toward both substrates is markedly higher than that shown by p38α *([11]*; see also legends for **Figs. 1** and **3B**).
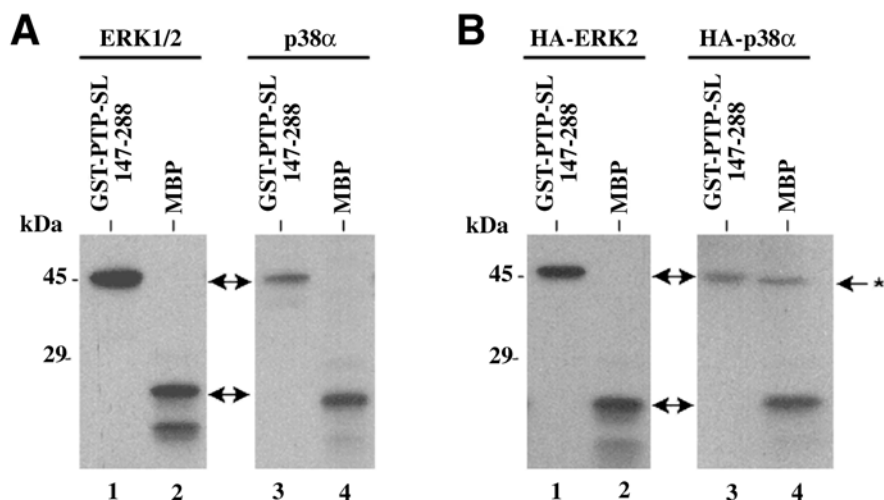
Fig. 1. Phosphorylation of GST-PTP-SL 147-288 and MBP by ERK1/2 and p38α. **(A)** Rat 1 cells were plated at semiconfluency and left for 6 h in the absence of fetal calf serum (FCS). Then, cells were stimulated with 10% FCS for 10 min (for ERK1/2; lanes 1 and 2) or with 0.5 *M* sorbitol for 20 min (for p38α, lanes 3 and 4) and lysed in lysis buffer. For each point, ERK1/2 and p38α were immunoprecipitated from cell lysates (0.75 mg of protein for ERK1/2, and 2.25 mg of protein for p38α) using specific anti-ERK1/2 or anti-p38α antibodies and protein A-Sepharose and subjected to immune complex in vitro kinase assays in the presence of equimolar amounts of GST-PTP-SL 147-288 (2 μg; lanes 1 and 3) or MBP (0.85 μg; lanes 2 and 4). **(B)** COS-7 cells were transfected with pCDNA3-HA-ERK2 or pECE-HA-MAPK (HA-p38α) in six-well plates using the DEAE-dextran method (1 μg of DNA/well). Cells were activated with 50 ng/mL of EGF for 5 min (for HA-ERK2; lanes 1 and 2) or with 0.5 *M* sorbitol for 20 min (for HA-p38α; lanes 3 and 4) and lysed. For each point, HA-ERK2 and HA-p38α were immunoprecipitated from cell lysates (from one well from a six-well plate for HA-ERK2, and from six wells from a six-well plate for HA-p38α) with the 12CA5 anti-HA monoclonal antibodies and protein A-Sepharose and subjected to in vitro kinase assays as in (A). For ERK1/2 and HA-ERK2, 2 μCi of [γ-^{32}P]ATP/ sample were used. For p38α and HA-p38α, 6 μCi of [γ-^{32}P]ATP/sample were used. Note that autophosphorylation of HA-p38α is observed in these assays (asterisk with an arrow in [B], lane 4), which could mask the phosphorylation of GST-PTP-SL 147-288. No autophosphorylation of HA-ERK2 is observed. Autoradiographs of representative experiments are shown. Since the in vitro specific activity of ERK1/2 is much higher than that of p38α, different amounts of cell lysates and [γ-^{32}P]ATP (see above), and different exposure times of the autoradiographs, were used for the assays with these two MAPKs. The exposure times were as follows: For (**A**), lanes 1 and 2 = 30 min; lanes 3 and 4 = 6 h. For (B), lanes 1 and 2 = 6 h; lanes 3 and 4 = 16 h. The relative expression of endogenous ERK1/2 and p38α in Rat 1 cells, and of recombinant HA-ERK2 and HA-p38α in COS-7 cells, was similar (not shown). All samples were analyzed by 15% SDS-PAGE under reducing conditions.

In the following protocol, GST-PTP-SL 147-288 is used both as the affinity reagent to precipitate ERK1/2 from cell lysates and as the substrate for these MAPKs in an in vitro kinase assay. The reliability and sensitivity of this method make it useful as an alternative to the method involving immunoprecipitation of ERK1/2 by antibodies, followed by immune complex in vitro kinase assay using MBP as the substrate *(12–14)*. In addition, a protocol is described to analyze the phosphoactive content of ERK1/2 by using GST-PTP-SL 147-288 and antiphosphoactive ERK1/2 antibodies. Examples are provided to illustrate the sensitivity and specificity of these assays in the measurement of the phosphorylation and activity of the ERK1/2 MAPKs.

2. Materials

All solutions are prepared in distilled/deionized/MilliQ filtered water. ^{32}P-radioactive materials should be handled with caution, following the safety laboratory standards for protection and disposal of radioactive waste.

2.1. Purification of GST-PTP-SL 147-288

1. *Escherichia coli* strain suitable for overexpression of GST-fusion proteins.
2. pGEX 5X-GST-PTP-SL 147-288 bacteria expression plasmid.
3. Luria-Bertani (LB) medium containing 50 μg/mL of ampicillin (Amp).
4. Isopropyl-β-D-thiogalactopyranoside (IPTG).
5. Glutathione-Sepharose.
6. Resuspension buffer: phosphate-buffered saline (PBS) pH 7.5, 0.1% Triton X-100.
7. Elution buffer: 50 mM Tris-HCl, pH 8.0, 10 mM glutathione.
8. Dialysis buffer: PBS; or Tris-buffered saline (TBS), pH 7.5.

2.2. Measurement of ERK1/2 Kinase Activities by In Vitro Kinase Assay

1. GST-PTP-SL 147-288 fusion protein, purified from *E. coli* according to standard protocols (*see* **Subheading 3.1.**).
2. Glutathione-Sepharose.
3. Lysis buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40 (IGEPAL CA-630); 2 mM Na$_3$VO$_4$, 100 mM NaF, 20 mM Na$_4$P$_2$O$_7$, 1 mM phenyhlmethyl-sulfonyl fluoride, 1 μg/mL of aprotinin.
4. Bradford protein concentration assay reagents.
5. Washing buffer: 20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerin, 10 mM Na$_4$P$_2$O$_7$.
6. Kinase reaction buffer: 20 mM HEPES, pH 7.5, 10 mM MgCl$_2$, 1 mM dithiothreitol (DTT), 2 mM Na$_3$VO$_4$, 3 μM cold ATP.
7. [γ-^{32}P] adenosine triphosphate (ATP).
8. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (2X): 100 mM Tris-HCl, pH 6.8, 4% bromophenol blue, 20% glycerin.

2.3. Measurement of Phosphoactive ERK1/2 by Western Blot

 1. GST-PTP-SL 147-288 fusion protein, purified from *E. coli* according to standard protocols (*see* **Subheading 3.1.**).
 2. Glutathione-Sepharose.
 3. Lysis buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40 (IGEPAL CA-630); 2 mM Na$_3$VO$_4$, 100 mM NaF, 20 mM Na$_4$P$_2$O$_7$, 1 mM phenyhlmethyl-sulfonyl fluoride; 1 µg/mL of aprotinin.
 4. Bradford protein concentration assay reagents.
 5. Washing buffer: 20 mM HEPES, pH 7.5; 150 mM NaCl; 0.1% Triton X-100; 10% glycerin, 10 mM Na$_4$P$_2$O$_7$.
 6. Polyvinylidene fluoride (PVDF) protein transfer membranes.
 7. Transfer buffer: 48 mM Tris base; 39 mM glycine, 0.037% SDS, 20% methanol.
 8. Prestained molecular weight standard markers.
 9. Ponceau solution: 2 g/L of Ponceau; 3% trichloroacetic acid, in H$_2$O.
10. Antiphosphoactive ERK1/2 and anti-ERK1/2 antibodies.
11. Peroxidase-conjugated secondary antibodies.
12. Incubation buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.05% Triton X-100, 0.25% gelatin.
13. Stripping solution: 65 mM Tris-HCl, pH 6.8, 2% SDS, 0.85% (v/v) 2-mercaptoethanol.
14. Western blotting chemiluminescence kit.

3. Methods

The methods described here involve the precipitation of soluble MAPKs from a cell lysate, using the GST-PTP-SL 147-288 substrate fusion protein as the affinity reagent, and glutathione-Sepharose as the solid phase to pull down the complexes. Following washing, an in vitro kinase assay is performed in the presence of [γ-^{32}P]ATP, using the pellets that contain the kinases and the GST-PTP-SL 147-288 fusion protein, which serves as the substrate. The phosphorylated GST-PTP-SL 147-288 protein (*Mr* = 45 kDa) is afterwards resolved by SDS-PAGE (*see* **Subheading 3.2.**). Alternatively, the pellets containing the MAPKs can be processed for phosphocontent analysis by immunoblot using antiphosphoactive MAP kinases (*see* **Subheading 3.3.**). As a support protocol, a standard procedure *(15)* for purification of GST-PTP-SL 147-288 is also provided (*see* **Subheading 3.1.**).

3.1. Purification of GST-PTP-SL 147-288

 1. Inoculate 25 mL of LB + Amp with one colony of *E. coli* transformed with pGEX 5X-GST-PTP-SL 147-288. Culture overnight (all cultures are made at 37ºC; *see* also **Note 1**).
 2. Inoculate 400 mL of LB + Amp with 10 mL of bacteria from the overnight culture. Grow to get an OD$_{600}$ of 0.6–0.8 (this takes about 2 h).

3. Add 40 μL of 1 *M* IPTG. Grow for another 4 h.
4. Centrifuge at 1200*g* for 20 min and discard the supernatant.
5. *Optional:* freeze at –20ºC the flask containing the bacteria pellet (*see* **Note 2**).
6. Resuspend in 5 mL of ice-cold resuspension buffer, avoiding excessive bubbling.
7. Transfer to a 50-mL plastic tube and sonicate (*see* **Note 3**).
8. Transfer to a Corex or a fast spin plastic tube and centrifuge at 6000*g* for 10 min at 4ºC.
9. Collect the supernatant and transfer to a 15-mL plastic tube. Add 0.5 mL of glutathione-Sepharose beads (50% beads in PBS).
10. Incubate for 30 min at 4ºC under rotation.
11. Wash three times with 10 mL of ice-cold PBS.
12. Elute three 1.5-mL fractions with ice-cold elution buffer. Keep on ice.
13. Analyze 10 μL of each fraction by SDS-PAGE and Coomasie staining (*Mr* of GST-PTP-SL 147-288 = 45 kDa).
14. Pool the appropriate fractions and dialyze in dialysis buffer. Make aliquots and freeze at –20ºC (*see* **Note 4**).

An example of the eluted fractions from a typical purification of GST-PTP-SL 147-288 is shown in **Fig. 2**. The migration of the GST-PTP-SL 147-288 protein is indicated by an arrow.

3.2. Measurement of ERK1/2 Kinase Activities by In Vitro Kinase Assay

1. Lyse cells in ice-cold lysis buffer (*see* **Notes 5** and **6**), and transfer the lysate to a 1.5-mL Eppendorf tube.
2. Centrifuge at 14,000*g* for 5 min, 4ºC. Save the supernatant and transfer to clean tubes.
3. *Optional:* Preclear the supernatant with 20–50 μL of glutathione-Sepharose for 30 min at 4°C under rotation. Centrifuge at 2500*g* for 1 min. Save the supernatant (*see* **Note 7**).
4. Measure the protein content using a Bradford assay.
5. Add to the supernatant 1 to 2 μg of GST-PTP-SL 147-288 for 2 h at 4ºC.
6. Add 20 μL of glutathione-Sepharose beads (1:1 in PBS) for 1 h at 4ºC under rotation (*see* **Note 8**).
7. Wash four times with 1 mL of ice-cold washing buffer.
8. Wash once with 1 mL of ice-cold reaction buffer (without cold ATP). Carefully aspirate the supernatant and leave the Eppendorf tube containing the pellet at room temperature for 1 to 2 min (*see* **Note 9**).
9. Add to the pellet 20 μL of room temperature reaction buffer containing 0.5–2 μCi of [γ-^{32}P]ATP for 20 min under constant shaking (*see* **Note 10**).
10. Add 30 μL of 2X SDS-PAGE loading buffer.
11. Boil the Eppendorf tube for 2 min, spin, and load the mix in a 10% SDS-PAGE gel. Include a lane with molecular weight standard markers.
12. Run the gel, stain with Coomasie blue, and dry on a piece of Whatman paper (*see* **Note 11**).
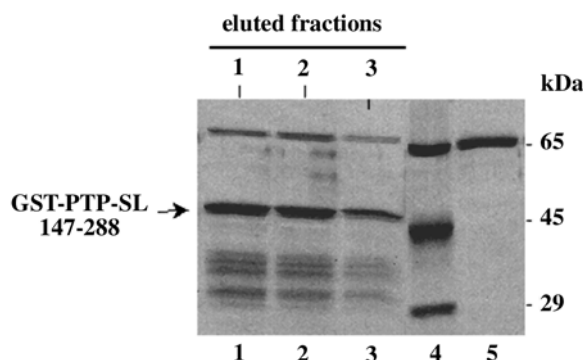13. Use the gel to expose an autoradiography film or a phosphorImager screen.

Fig. 2. Purification of GST-PTP-SL 147-288 from *E. coli*. The fusion protein was purified from 400 ml of *E. coli* transformed with pGEX-5X-GST-PTP-SL 147-288, as described in **Subheading 3.1.** Ten microliters of the eluted fractions (lanes 1–3) were analyzed by 10% SDS-PAGE under reducing conditions and Coomasie staining. The migration of GST-PTP-SL 147-288 is indicated with an arrow. Molecular weight (lane 4) and bovine serum albumin (BSA) standards (2 μg; lane 5) are shown for comparison. The band around 65 kDa in lanes 1–3 represents an unidentified bacterial contaminant, which does not interfere with the kinase assays.

14. For quantification of incorporated counts per minute, scise out the GST-PTP-SL 147-288 (45 kDa) band from the dry gel and put it into an Eppendorf tube containing 1 mL of scintillation liquid. Count on a scintillation counter. Alternatively, dry gels can be processed for quantification using a phosporImager

An example of a kinase assay using the GST-PTP-SL 147-288 fusion protein as the affinity reagent and the substrate for ERK1/2 is shown in **Fig. 3A,B** (top panel; *see* also **Note 12**). As observed, phospholabeling of GST-PTP-SL 147-288 is inhibited when cells are preincubated in the presence of the MEK1/2 inhibitor PD98059, but not in the presence of the p38α inhibitor SB203580. **Figure 3B** also shows a comparison of kinase assays using the GST-PTP-SL 147-288 fusion protein (top panel), anti-ERK1/2 (middle panel) or anti-p38α (bottom panel) antibodies to precipitate the MAPKs, and GST-PTP-SL 147-288 as the substrate. As shown, the pattern of phosphorylation of GST-PTP-SL 147-288 after precipitation of MAPKs with this fusion protein is identical to the pattern obtained after immunoprecipitation with anti-ERK1/2 antibodies. The sensitivity of the kinase assay using GST-PTP-SL 147-288 as the affinity reagent is illustrated in **Fig. 4**. Efficient phospholabeling of the fusion protein was obtained after precipitation of the MAPKs from amounts of total cell lysates as low as 4 μg of protein.
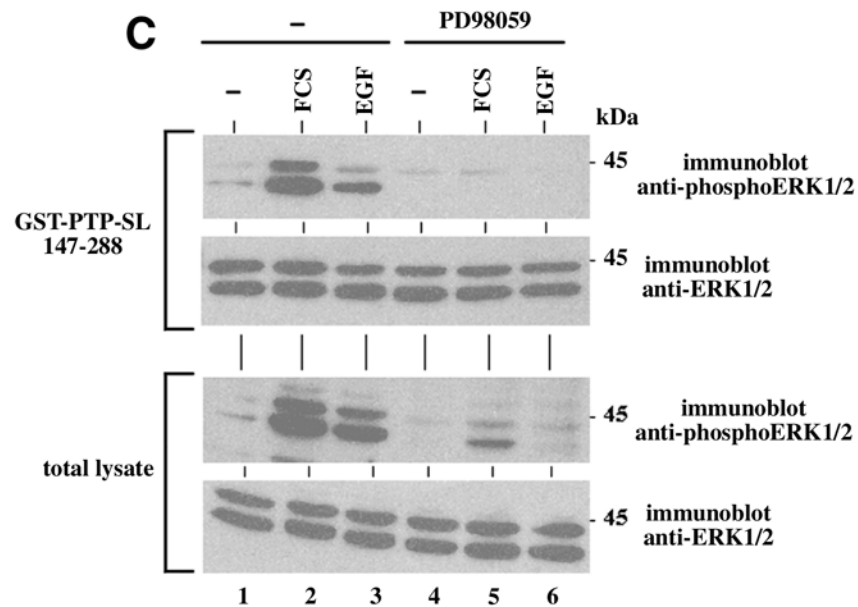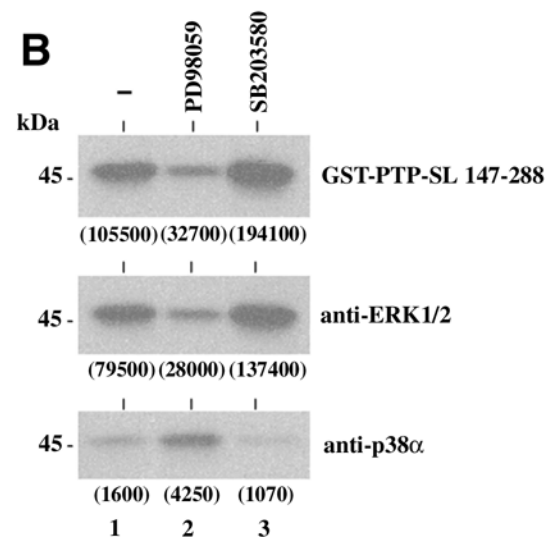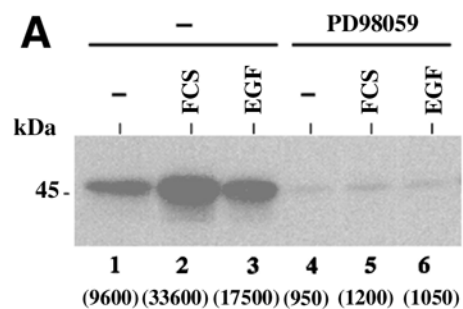
A

	–		PD98059		
–	FCS	EGF	–	FCS	EGF

kDa

45 -

1 2 3 4 5 6
(9600) (33600) (17500) (950) (1200) (1050)

B

–	PD98059	SB203580

kDa

45 - GST-PTP-SL 147-288
(105500) (32700) (194100)

45 - anti-ERK1/2
(79500) (28000) (137400)

45 - anti-p38α
(1600) (4250) (1070)

1 2 3

C

	–		PD98059		
–	FCS	EGF	–	FCS	EGF

kDa

GST-PTP-SL 147-288

45 - immunoblot anti-phosphoERK1/2

45 - immunoblot anti-ERK1/2

total lysate

45 - immunoblot anti-phosphoERK1/2

45 - immunoblot anti-ERK1/2

1 2 3 4 5 6

Fig. 3.

3.3. Measurement of Phosphoactive ERK1/2 by Western Blot

1. Lyse cells in ice-cold lysis buffer (*see* **Notes 5** and **7**), and transfer the lysate to a 1.5-mL Eppendorf tube.
2. Centrifuge at 14,000*g* for 5 min, 4ºC. Save the supernatant and transfer to clean tubes.
3. *Optional:* Preclear the supernatant with 20–50 µL of glutathione-Sepharose for 30 min at 4ºC under rotation. Centrifuge at 3000 rpm for 1 min. Save the supernatant (*see* **Note 7**).
4. Measure the protein content using a Bradford assay.
5. Add to the supernatant 1 to 2 µg of GST-PTP-SL 147-288 for 2 h at 4ºC.
6. Add 20 µL of glutathione-Sepharose beads (1:1 in PBS) for 1 h at 4ºC under rotation (*see* **Note 8**).
7. Wash once with 1 mL of ice-cold PBS.
8. Add 40 µL of 2X SDS-PAGE loading buffer.
9. Boil the Eppendorf tube for 2 min, spin, and load the mix in the SDS-PAGE gel. Include a lane with prestained molecular weight standard markers (*see* also **Note 13**).

Fig. 3. *(previous page)* Measurement of ERK1/2 activities using GST-PTP-SL 147-288. **(A)** Rat 1 cells were plated at semiconfluency and left for 6 h in the absence of FCS. Then, cells were left untreated (lanes 1 and 4) or were incubated for 10 min in the presence of 10% FCS (lanes 2 and 5) or 50 ng/mL of EGF (lanes 3 and 6). In lanes 4–6, cells were preincubated for 30 min with the MEK1/2 inhibitor PD98059 (30 µ*M*), before adding the stimuli. Cells were lysed, lysates (0.75 mg of protein/point) were precipitated with 1.5 µg of GST-PTP-SL 147-288, and pellets were subjected to in vitro kinase assay, as described in **Subheading 3.2.** Autoradiography of a representative experiment is shown. **(B)** Rat 1 cells were plated at semiconfluency and stimulated with 10% FCS after starvation, as in (A), in the absence of inhibitors (lane 1), or after a 30-min preincubation in the presence of the MEK1/2 inhibitor PD98059 (30 µ*M*) (lane 2) or the p38α inhibitor SB203580 (10 µ*M*) (lane 3). Cell lysates (0.75 mg of protein/point) were precipitated with GST-PTP-SL 147-288 (1.5 µg/sample) (top panel) or were immunoprecipitated with specific anti-ERK1/2 antibodies (middle panel) or anti-p38α antibodies (bottom panel). In the top panel, the pellets were processed for kinase assays as in (A); in the middle and bottom panels, 1.5 µg of GST-PTP-SL 147-288 was added to the pellets as the substrate. Numbers in parentheses at the bottom of the panels in (A) and (B), correspond to the radioactivity incorporated (in counts per minute) into the GST-PTP-SL 147-288 substrate in the distinct samples. **(C)** Rat 1 cells were plated at semiconfluency and treated with stimuli in the absence (lanes 1–3) or in the presence of the MEK1/2 inhibitor PD98059 (lanes 4–6), as in (A). In the two top panels, cell lysates (2 mg of protein/point) were precipitated with 1.5 µg of GST-PTP-SL 147-288; and in the two bottom panels, total lysate samples were loaded (100 µg of protein/point). Filters were analyzed for phosphocontent of ERK1/2 by immunoblot using an antiphosphoactive-ERK1/2 antibody, as indicated. ERK1/2 total protein content was analyzed after stripping of the filters and reprobing with anti-ERK1/2 antibodies, as indicated. All samples were resolved by 10% SDS-PAGE under reducing conditions.
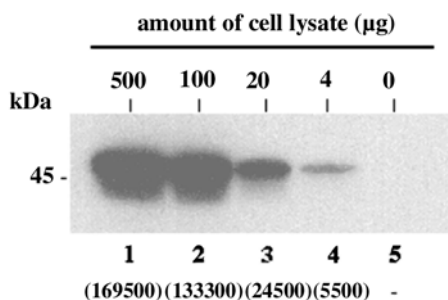
Fig. 4. Sensitivity of the ERK1/2 kinase assay using GST-PTP-SL 147-288. Rat 1 cells were plated at semiconfluency and stimulated, after 6 h of starvation, with 10% FCS for 10 min. Cells were lysed and the indicated amounts of cell lysate protein were mixed with 0.3 mL of PBS containing 1 mg/mL of BSA (as a carrier protein), precipitated with GST-PTP-SL 147-288 (1.5 µg/sample), and processed for kinase assays as described in **Subheading 3.2.** Numbers in parentheses at the bottom correspond to the radioactivity incorporated (in counts per minute) into the GST-PTP-SL 147-288 substrate band in each sample. Samples were analyzed by 10% SDS-PAGE under reducing conditions.

10. Run the gel and transfer to the PVDF membrane. Cut a piece of the membrane in the range of 30–60 kDa (*see* **Note 14**).
11. *Optional:* Stain the membrane with Ponceau to check the transfer and the presence of the GST-fusion proteins.
12. Detect the phosphoactive ERK1/2 (about 42–44 kDa) by standard Western blot and chemoluminiscence techniques, using an antiphosphoactive ERK1/2 antibody as the primary antibody.
13. Strip the membrane by incubating in stripping solution at 50ºC for 1 h, and detect the total content of ERK1/2 by reprobing it with an anti-ERK1/2 antibody (*see* **Note 15**).

An example of the detection of phosphoactive ERK1/2 using the GST-PTP-SL 147-288 fusion protein as the affinity reagent, and antiphosphoactive ERK1/2 antibodies, is shown in **Fig. 3C**, in comparison with that detected from total cell lysates.

4. Notes

1. The described protocol involves a batch purification of the GST-fusion protein, using 15-mL centrifuge polypropylene tubes (V-shaped bottom). If large amounts of fusion protein are going to be purified, the use of a column containing the glutathione-Sepharose beads is recommended; a bed of 1 to 2 mL of glutathione-Sepharose beads can be easily prepared in a 10-mL plastic syringe, which is suitable for purifications from 400 to 800 mL of bacteria culture. After elution of the

fusion protein and washing, the glutathione-Sepharose can be stored in PBS containing 0.02% sodium azide.

2. After **step 4**, the bacteria pellet can be frozen at –20ºC, so the following steps can be performed the next day.

3. Sonication should be performed according to the specifications of the sonication apparatus. It is recommended that the sonication be performed maintaining the sample on ice and applying several short pulses. After sonication, the suspension should get clarified.

4. To perform precipitation of MAPKs with GST-PTP-SL 147-288, followed by kinase assays (*see* **Subheading 3.2.**), use PBS or TBS to dialyze the fusion protein after its purification, as indicated in **Subheading 2.1.** The GST-PTP-SL 147-288 fusion protein can also be used as a substrate for the MAPKs after immunoprecipitation with specific antibodies (as shown in **Figs. 1** and **3B**); in such a case dialyze the fusion protein in 20 m*M* HEPES, pH 7.5; 10 m*M* MgCl$_2$; and 1 m*M* DTT.

5. For adherent cells, rinse the plate with ice-cold PBS and lyse the cells directly on the plate; use 1 mL of lysis buffer for one 10-cm-diameter plate, or 0.25 mL/well for a six-well plate. For nonadherent cells, centrifuge the cells, wash with ice-cold PBS, and lyse the cell pellets; use 1 mL of lysis buffer for 10×10^6 cells.

6. To prevent protein degradation and/or inactivation of the MAPKs, it is important to include in the lysis buffer a cocktail of protease and phosphatase inhibitors (freshly added), as indicated in **Subheading 2.2.**

7. Preclearing may help to reduce undesired background of nonspecific kinase activity.

8. To pipet small volumes of glutathione-Sepharose beads, it is convenient to cut out the end of the micropipet tip.

9. Washing with reaction buffer that lacks cold ATP is recommended to prevent phosphorylation of the substrate at this step.

10. The concentrations of radiolabeled and cold ATP in the reaction buffer may be changed to fit the desired conditions of the reaction.

11. Staining of the gel with Coomassie blue allows monitoring of the presence of equal content of the GST-PTP-SL 147-288 substrate in all lanes, as well as visualization of the substrate band that will be excised out of the gel if counts per minute are to be determined.

12. GST-PTP-SL 147-288 precipitates both ERK1/2 and p38α *(11)*; however, since the activity of ERK1/2 in these in vitro kinase assays considerably exceeds the activity of p38α (see legends for **Figs. 1** and **3B**), the incorporation of radioactivity to the substrate under these conditions is almost exclusively due to ERK1/2. Controls of specificity can be run in parallel by using specific inhibitors of the ERK1/2 (PD98059) *(16)* or the p38α (SB203580) *(17)* pathways, as shown in **Fig. 3B**. As observed, the phospholabeling of GST-PTP-SL 147-288 after precipitation and in vitro phosphorylation using the method described here, correlates with that obtained when ERK1/2 are immunoprecipitated with specific antibodies and then subjected to kinase assays in the presence of the substrate. However, immunoprecipitated p38α yields a distinct pattern of activity.

13. Following precipitation with GST-PTP-SL 147-288 the phosphoactive content of ERK1/2 or p38α can be monitored, using antiphosphoactive ERK1/2- or p38α-specific antibodies (*see* **Fig. 3C**).

14. Running prestained molecular weight standard markers allows a strip of transfer membrane containing the MAPKs bands to be cut.

15. Reprobing with an anti-ERK1/2 or anti-p38α antibody is recommended to monitor that equal amounts of MAPKs are present in all lanes.

Acknowledgments

References

1. Sharrocks, A. D., Yang, S.-H., and Galanis, A. (2000) Docking domains and substrate-specificity determination for MAP kinases. *Trends Biochem. Sci.* **25,** 448–453.
2. Adler, V., Franklin, C. C., and Kraft, A. S. (1992) Phorbol esters stimulate the phosphorylation of c-Jun but not v-Jun: regulation by the N-terminal δ domain. *Proc. Natl. Acad. Sci. USA* **89,** 5341–5345.
3. Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993) Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes Dev.* **7,** 2135–2148.
4. Raingeaud, J., Gupta, S., Rogers, J. S., et al. (1995) Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J. Biol. Chem.* **270,** 7420–7426.
5. LoGrasso, P. V., Frantz, B., Rolando, A. M., O'Keefe, S. J., Hermes, J. D., and O'Neill, E. A. (1997) Kinetic mechanism for p38 MAP kinase. *Biochemistry* **36,** 10,422–10,427.
6. Cobb, M., Boulton, T. G., and Robbins, D. J. (1991) Extracellular signal regulated kinases: ERKs in progress. *Cell Regul.* **2,** 965–978.
7. Davis, R. J. (1993) The mitogen-activated protein kinase signal transduction pathway. *J. Biol. Chem.* **268,** 14,553–14,556.
8. Erickson, A. K., Payne, M., Martino, P. A., et al. (1990) Identification by mass spectrometry of threonine 97 in bovine myelin basic protein as a specific phosphorylation site for mitogen-activated protein kinase. *J. Biol. Chem.* **265,** 19,728–19,735.
9. Sanghera, J. S., Aebersold, R., Morrison, H. D., Bures, E. J., and Pelech, S. L. (1990) Identification of the sites in myelin basic protein that are phosphorylated by meiosis-activated protein kinase p44*mpk*. *FEBS Lett.* **273,** 223–226.
10. Pulido, R., Zúñiga, Á., and Ullrich, A. (1998) PTP-SL and STEP protein tyrosine phosphatases regulate the activation of the extracellular signal-regulated kinases ERK1 and ERK2 by association through a kinase interaction motif. *EMBO J.* **17,** 7337–7350.
11. Zúñiga, Á., Torres, J., Úbeda, J., and Pulido, R. (1999) Interaction of mitogen-activated protein kinases with the kinase interaction motif of the tyrosine phos-

phatase PTP-SL provides substrate specificity and retains ERK2 in the cytoplasm. *J. Biol. Chem.* **274,** 21,900–21,907.

12. Alessi, D. R., Cohen, P., Ashworth, A., Cowley, S., Leevers, S. J., and Marshall, C. J. (1995) Assay and expression of mitogen-activated protein kinase, MAP kinase kinase, and Raf. *Method Enzymol.* **255,** 279–290.

13. Reuter, C. W. M., Catling, A. D., and Weber, M. J. (1995) Immune complex kinase assays for mitogen-activated protein kinase and MEK. *Method Enzymol.* **255,** 245-256.

14. Rubinfeld, H. and Seger, R. (1998) Detection of MAP kinase signaling in *Current Protocols in Cell Biology* (Bonifacio, J. M., Dasso, M., Harford, J. B., et al., eds.) John Wiley & Sons, New York, pp. 14. 3.1–14.3.19.

15. Smith, D. B. and Johnson, K. S. (1998) Single-step purification of polypeptides expressed in *Escherichia Coli* as fusions with glutathione *S*-transferase. *Gene* **67,** 31–40.

16. Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Saltiel, A. R. (1995) A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl. Acad. Sci. USA* **92,** 7686–7689.

17. Cuenda, A., Rouse, J., Doza, Y. N., et al. (1995) SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.* **364,** 229–233.

4

Activation of SAPKs/JNKs and p38s In Vitro

John M. Kyriakis, Hong Liu, and Deborah N. Chadee

1. Introduction

The stress-activated protein kinases (SAPKs)/c-Jun N_2-terminal kinases (JNKs) and the p38 mitogen-activated protein kinases (MAPKs) are two MAPK groups activated predominantly by stresses and proinflammatory stimuli. **Table 1** lists some of the stimuli known to recruit both the SAPKs/JNKs and p38s. In general, the same stimuli that activate the SAPKs/JNKs will also activate the p38s. One exception is ischemia-reperfusion. In this instance, the SAPKs/JNKs are activated exclusively during reperfusion, while the p38s are activated during ischemia and remain active during reperfusion *(1)*.

The SAPKs/JNKs and p38s are potent upstream activators of the activator protein-1 (AP-1) transcription factor (**Table 2**). AP-1 is a heterodimer that consists typically of c-Jun complexed with a member of the c-*fos* or activating transcription factor (ATF) family. The SAPKs/JNKs regulate AP-1 by phosphorylating and activating the *trans*-activating function of c-Jun and ATF2, and by phosphorylating and activating Elk1, a component of the serum response factor transcription factor that *trans* activates c-*fos*. Once activated, AP-1 *trans* activates numerous stress- and mitogen-regulated genes *(1)*. Genetic studies indicate that consistent with their central role in the regulation of gene expression, mammalian SAPKs/JNKs are important in neuronal development, CD4^{+} T-helper cell differentiation, T-cell activation, and the proapoptotic response to genotoxins *(1)*. Genetic studies indicate that the p38s are important in the maintenance of placental sufficiency and embryonic hematopoiesis through the regulation of erythropoietin production *(2,3)*.

The p38s can also phosphorylate and activate a number of Ser/Thr kinases including MAPK-activated protein kinase-2 (MAPKAP-K2), p38-activated and regulated kinase (PRAK), and MAPK and SAPK-regulated kinase (MSK). These downstream kinases can phosphorylate and activate transcription fac-

Table 1
Representative Stimuli That Activate SAPK/JNK and p38 In Vivo[a]

Stimulus (ref.)	Target kinase(s)	Sensitive cell lines	Amount used	Comments
Anisomycin (6)	SAPK/JNK, p38	All cells	1-10 µg/mL, 1–60 min	
UV-C (12)	SAPK/JNK, p38	All adherent cells	40 J/m^2, 20 s^{-1}	Drain medium from cells, and wash with PBS before exposure.
γ Radiation (13)	SAPK/JNK, p38	All cells (see comments)	10–20 gy, ^{137}Cs source at 0.76 gy/min	This is controversial and has not been replicated in some laboratories. After treatment, wait between 20 min and 4 h before harvest.
cis platinum (CDDP) or cytosine arabinoside (ara-C) (13)	SAPK/JNK, p38	All cells	50–300 µM, 2–6 h	
Methyl methane sulfonate (MMS) (13)	SAPK/JNK, p38	All cells	1 mM, 3 h	
Oxidant stress (H$_2$O$_2$) (9)	SAPK/JNK, p38	All cells	2–20 mM, 2–60 min	
TNF (6)	SAPK/JNK, p38	HepG2, CCD18-CO, HEK293, COS, U937, HL-60, Jurkat, L929, HeLa, primary thymocytes or hepatocytes	2–100 ng/mL, 1–60 min	Try to pick a cell type in which TNF is known to induce a biologic effect.

IL-1 *(6)*	SAPK/JNK, p38	EL-4, HepG2, Jurkat, HL-60, KB	2–100 ng/mL, 1–60 min	Try to pick a cell type in which IL-1 is known to induce a biologic effect.
Endothelin *(14,15)*	SAPK/JNK, p38	Endothelial, mesangial, pulmonary fibroblast, vascular smooth muscle, cardiomyocyte	10–200 nM, 2–60 min	Try to pick a cell type in which endothelin is known to induce a biologic effect.
Angiotensin II *(14)*	SAPK/JNK	Endothelial, mesangial, pulmonary fibroblast, vascular smooth muscle, cardiomyocyte, hepatoma	20–500 nM, 2–60 min	Try to pick a cell type in which the type II antiotensin II receptor is expressed and in which angiotensin II is known to induce a biologic effect.
Ischemia/ reperfusion *(16)*	SAPK/JNK, p38	Kidney, heart, brain	40 min ischemia, 2–120 min reperfusion	SAPK/JNK is activated only during reperfusion.

63

[a]This table is meant to be a guide for stimuli that recruit SAPK/JNK and p38. The lists of stimuli and cells used are in no way inclusive. The conditions for stimulation are also meant to serve as a guide. We strongly recommend that time courses and dose responses be performed whenever possible.

Table 2
Some Representative Substrates of SAPKs/JNKs and p38s[a]

Substrate	Function	Upstream activator(s)
c-Jun	Transcription factor, component of AP-1	SAPK/JNK
ATF2	Transcription factor, component of AP-1	SAPK/JNK, p38
Elk-1	Transcription factor, component of ternary complex factor	SAPK/JNK (and ERK)
SAP1a	Transcription factor, component of ternary complex factor	p38
CHOP/GADD153	Transcription factor, regulator of DNA damage and stress response	p38
MAPKAP kinases-2,3	Ser/Thr kinases, regulate small heat-shock protein Hsp27, and TNF message translation	p38 (α and β isoforms only)
PRAK	Ser/Thr kinase, regulates small heat-shock protein Hsp27	p38 (α and β isoforms only)
MSK	Ser/Thr kinase, regulates chromatin remodeling and CREB phosphorylation	p38 (α and β isoforms only) (and ERK)
MNKs	Ser/Thr kinases, regulate translation	p38 (α and β isoforms only) (and ERK)

[a]Reviewed in **ref. 1**. This table is not inclusive and is meant to illustrate the diversity of SAPK/JNK and p38 substrates.

tors (thus, MSK is a potent activator of cyclic adenosine monophosphate response element binding protein [CREB]), and cytosolic components (MAPKAP-K2 and PRAK phosphorylate the small heat-shock protein Hsp27, and MAPKAP-K2 can phosphorylate key elements in the signaling pathway by which bacterial lipopolysaccharide stimulates enhanced translation of tumor necrosis factor [TNF] mRNA) *(1)* (**Table 2**).

As with all MAPKs, the SAPKs/JNKs and p38s are proline-directed kinases; however, their substrate specificity is dictated in large part by the presence of specific docking sites on the substrates. These docking motifs interact with

regions of the SAPK/JNK or p38 polypeptides allowing for specific high-affinity interactions between enzyme and substrate. Polybasic docking motifs are present on many protein kinase substrates of the p38s. These bind to the recently identified common docking (CD) domain, an extracatalytic region rich in acidic residues, found in all MAPKs *(1)*. In contrast to the basic loop–CD domain interaction, certain transcription factor substrates of the SAPKs/JNKs (e.g., c-Jun) or the p38s (e.g., ATF2) contain a hydrophobic pocket (in c-Jun this corresponds to the δ domain) that interacts with a region spanning subdomains IX and X of the kinase domain substrate binding loop. Again, this interaction confers a high degree of substrate specificity on the SAPKs/JNKs or p38s *(1)*. **Table 2** provides representative SAPK/JNK and p38 substrates.

The SAPKs/JNKs are encoded by three genes: SAPKα/JNK2, SAPKβ/JNK3, and SAPKγ/JNK1 (**Table 3**). SAPKβ/JNK3 expression is restricted largely to brain, heart, and testis, while the other SAPK/JNK isoforms are expressed ubiquitously. Although subtle differences in substrate specificity exist among SAPK/JNK isoforms, the significance of these differences is nebulous *(1)*.

Four p38 genes are known: p38α, p38β/p38-2, p38γ/SAPK3/ERK6, and p38δ/SAPK4 (**Table 3**). These kinases differ more significantly in their substrate specificity than do the SAPK/JNK isoforms. Thus, p38α and β, but not γ or δ, can phosphorylate the p38 protein kinase substrates (MAPKAP-K2, PRAK, MSK), while all four isoforms can phosphorylate the transcription factor substrates *(1)*.

Two MEKs are known to lie upstream of the SAPKs/JNKs: SAPK/ERK-kinase-1/MAPK-kinase-4 (SEK1/MKK4) and MKK7. Genetic and biochemical studies indicate that MKK7 is activated by all stimuli that recruit the SAPKs/JNKs, while SEK1/MKK4 is activated by a more restricted spectrum of agonists that includes certain environmental stresses and, to a lesser extent, inflammatory cytokines *(1)*.

Similarly, two MEKs, MKK3 and MKK6, lie upstream of the p38s. MKK3 is regulated in a manner similar to that of SEK1/MKK4 and is recruited most strongly by environmental stresses. MKK6 is activated by all known p38 agonists *(1)*.

At least a dozen Ser/Thr kinases falling into several protein kinase families have been implicated as MAP3Ks upstream of the SAPKs/JNKs and p38s. These include the MEK kinases (MEKKs), which bear a structural similarity within their catalytic domains to *STE11* a MAP3K of the pheromone response pathway of *Saccharomyces cerevisiae*; the mixed lineage kinases (MLKs), which are structurally similar to both Ser/Thr and Tyr kinases; and the thousand-and-one kinases (TAOs), which bear similarities to *STE20* of the pheromone response pathway of *S. cerevisiae (1)*. **Table 4** provides MAP3Ks upstream of the SAPKs/JNKs and p38s.

Table 3
SAPK/JNK and p38 Nomenclature[a]

Name	Alternate names
SAPK-α	JNK2, SAPK1a
SAPK-p54α1	JNK2β2
SAPK-p54α2	JNK2α2
SAPK-p46α1	JNK2β1
SAPK-p46α2	JNK2α1
SAPK-β	JNK3, SAPK1β
SAPK-p54β1	JNK3β2
SAPK-p54β2	JNK3α2
SAPK-p46β1	JNK3β1
SAPK-p46β2	JNK3α1
SAPK-γ	JNK1, SAPK1c
SAPK-p54γ1	JNK1β2
SAPK-p54γ2	JNK1α2
SAPK-p46γ1	JNK1β1
SAPK-p46γ2	JNK1α1
p38α	SAPK2a, CSBP1
p38β	SAPK2b, p38-2
p38γ	SAPK3 ERK6
p38δ	SAPK4

[a]Reviewed in **ref. *1***.

This chapter focuses on assays for upstream activators of the SAPKs and p38s. We will discuss biochemical assays of MEKs (with a focus on SEK1/MKK4) and MAP3Ks (with MEKK1 serving as the paradigm). These assays were developed in our laboratory to be versatile, enabling the investigator to assay similar enzymes using the same technique. Accordingly, each assay is described for a single enzyme; however, these methods can be adapted with little difficulty to other MAPK systems.

2. Materials

2.1. Buffers

1. Buffer 1: 20 m*M* HEPES (pH 7.4), 2 m*M* EGTA, 50 m*M* β-glycerophosphate (Ser/Thr phosphatase inhibitor), 1 m*M* dithiothreitol (DTT), 1 m*M* Na$_3$VO$_4$ (Tyr phosphatase inhibitor, from a 20 m*M* stock boiled in a microwave for 2 min, right before adding to the buffer and cooled to room temperature), 1% Triton X-100 (w/v), 10% (v/v) glycerol; plus the following protease inhibitors: 2 μ*M* leupeptin, 10 kallikrein-inhibiting U/mL of Trasylol (also called aprotinin), 2 μ*M* pepstatin,

Table 4
Stress-Regulated MAP3K Nomenclature[a]

Name	Alternate names	Substrates/effectors
MEKK1	—	SEK1, MKK7
MEKK2	—	SEK1, MEK1
MEKK3	—	SEK1, MEK1, ?MKK3, ?MKK6
MEKK4	MAP three kinase-1 (MTK1)	SEK1, MKK3, MKK6
ASK1	MAPK kinase-kinase-5 (MAPKKK5)	SEK1, MKK3, MKK6
TAK1	—	SEK1, MKK3, MKK6
Tpl-2 (in rats)	Cot (in humans)	MEK1, SEK1
MLK2	MST	SEK1, MKK7
MLK3	SPRK, PTK1	SEK1, MKK7
DLK	MUK, ZPK	SEK1, MKK7
TAO1/2	Prostate-specific kinase (PSK, a splicing isoform of TAO2)	MKK3, ?SEK/MKK7 (PSK only)

[a]Reviewed in **ref. 1**.

and 400 μM pneylmethylsulfonyl fluoride (PMSF). Add the PMSF from a 400 mM stock prepared in methanol immediately before harvesting the cells. This buffer is for lysis of transfected cells or cells from which the endogenous kinase of interest is to be isolated and assayed *(4,5)*. We generally prepare a 10X base stock solution consisting of the HEPES, EGTA, and β-glycerophosphate. This can be stored indefinitely at 4°C. The pH of the stock can be adjusted; however, the pH of the buffer should be readjusted immediately prior to addition of the PMSF.

a. Triton X-100 comes from a 10% (w/v) stock that can be stored indefinitely at 4°C.

b. DTT is prepared as a 1000X stock in water, aliquoted (1 mL), snap frozen on liquid nitrogen (or dry ice–methanol), and stored at –80°C. DTT stock is stable indefinitely at –80°C.

c. Na$_3$VO$_4$ comes from a 20 mM stock. Over time, Na$_3$VO$_4$ in stock solutions tends to form inactive aggregates *(6)*. Accordingly, we typically take ~25 mL of stock and boil it in a microwave for 30 s to 1 min and cool to room temperature. The necessary amount of Na$_3$VO$_4$ is then added to the buffer from this boiled preparation.

d. Leupeptin, Trasylol/aprotinin, and pepstatin are, at best, weak protease inhibitors and are added to bolster the effects of PMSF (*see* next item). Leupeptin and Trasylol/aprotinin are prepared as 1000X stocks in water. Pepstatin is prepared as a 1000X stock in 90% (v/v) methanol.

e. PMSF is prepared as a 1000X stock in methanol. It is extremely toxic and care should be taken to avoid contact with skin or eyes. The half-life of PMSF in aqueous solutions is short (in practical terms ~30 min). Thus, it should only be added last, immediately before one plans to harvest the cells.

    f. The final buffer can only be used once and must be prepared freshly for each experiment.

2. Buffer 2: 20 m*M* Tris-HCl (pH 7.4), 2 m*M* EGTA, 250 m*M* sucrose (preserves isoosmotic conditions without affecting ionic strength), 1 m*M* DTT: plus the following protease inhibitors: 2 μ*M* leupeptin, 10 kallikrein-inhibiting U/mL of Trasylol (aprotinin), and 400 μ*M* PMSF. This is a buffer for preparation of glutathione-*S*-transferase (GST)-tagged proteins that are expressed in mammalian cells (COS or, preferably, 293 cells) from the pEBG vector. However, this buffer can also be used to prepare proteins expressed in Sf9 cells from baculoviral constructs; and to prepare extracts from which one can isolate proteins that are not epitope tagged. The buffer contains no detergent; accordingly, it is suitable only for proteins that are cytosolic. We use this buffer for preparation of some recombinant MEKs (GST-SEK1/MKK4, GST-MKK6) *(7)*.

    a. If protein Ser/Thr phosphorylation needs to be preserved, include 50 m*M* β-glycerophosphate in the buffer *(2)*.

    b. If Tyr phosphorylation needs to be preserved, add Na_3VO_4 to 0.2 m*M* (from a boiled preparation as described in **item 1**). However, the vanadate should not be added until all membraneous organelles have been removed by centrifugation. Vanadate disrupts transmembrane ion potentials and leads to the rupture of membranous organelles including mitochondria. Accordingly, the preparation will be unnecessarily contaminated with proteins from these organelles if vanadate is added before centrifugation *(6,8)*. As long as the temperature of the preparation is kept at 4°C, addition of the vanadate at this apparently late time will not adversely affect most Tyr phosphorylation—especially that of MAPKs.

    c. If one's studies call for the chromatography of cell extracts on columns (ion exchange or affinity, but not hydrophobic), Triton X-100 can be added to 0.1% (w/v) to the cell extract after centrifugation. This will help minimize fixed losses on columns owing to hydrophobic effects. Addition of detergent before centrifugation will lead to leakage of cell compartments and unnecessary contamination of the extract *(8)*. If one's plans call for hydrophobic chromatography, however, avoid adding any detergent until all hydrophobic chromatography is complete.

    d. As with buffer 1, a base stock of Tris and EGTA can be prepared and stored at 4°C; however, the final pH of the buffer should be adjusted immediately prior to addition of the PMSF. All other stocks are prepared and handled as for buffer 1. The final buffer can be used only once and must be prepared freshly for each isolation.

    e. We also prepare this buffer, without sucrose, in 50% (v/v) glycerol, 0.1% (w/v) Triton X-100 instead of water. This is a storage buffer into which purified, expressed proteins are dialyzed for storage at –20°C.

3. Buffer 3: 100 m*M* Tris-HCl (pH 7.6), 2 m*M* EGTA, 0.1% Triton X-100, 1 m*M* DTT, 0.5–1 *M* LiCl or NaCl (optional—or as needed). This is a buffer for washing immunoprecipitates stringently prior to kinase assay. We have included LiCl

or NaCl, which enables a high ionic strength wash, as an option. One may find, however, that associated polypeptides improve certain aspects of the kinase assay (a source for further scientific investigation!). In addition, if one is attempting to preserve protein-protein interactions, we suggest reducing the stringency of or eliminating the high LiCl/NaCl wash. Typically for coimmunoprecipita-tions, we use 50 m*M* NaCl; however, for simple assays of SAPK/JNK or SEK1/MKK4, we wash with 0.5–1 *M* LiCl *(4,5,7)*. The high-stringency wash is also excellent for washing the immobilized GST proteins (from bacteria; or eukaryotic sources) during purification *(4,5,7)*. For MAP3K assays, the situation is more complex— Raf-1 was best assayed after stringent washing *(4)*; however, stress-regulated MAP3Ks (e.g., MEKK1, ASK1) require more gentle washings to enable detection of in vivo activation ([**ref. 9**]; unpublished findings). As with buffers 1 and 2, this buffer can be prepared from a base stock of Tris-EGTA (10X, stored at 4˚C). The remaining stocks are prepared as for buffers 1 and 2. LiCl or NaCl can be added in dry form or from 5 *M* stocks. Readjust the pH after preparation of the buffer. The completed buffer can be used only once and should be prepared freshly for each experiment.

4. Buffer 4: 20 m*M* MOPS (pH 7.2), 2 m*M* EGTA, 1 m*M* DTT, 0.1% Triton X-100. This is a simple protein kinase assay buffer *(8)*. Note that the buffer does not contain adenosine triphosphate (ATP) or Mg, which allows for the addition of these at any desired concentration. We generally prepare this buffer in its final form, aliquot (2 mL), snap freeze (liquid nitrogen or dry ice–methanol), and store at –80˚C. It keeps indefinitely under these conditions.

5. Buffer 5: 50 m*M* Tris-HCl (pH 7.8), 60 m*M* NaCl, 2 m*M* DTT, 1 m*M* EDTA, 4 m*M* benzamidine (weak protease inhibitor), 0.05% (w/v) Triton X-100; pthe following additional protease inhibitors: 2 µ*M* leupeptin, 2 µ*M* pepstatin, 10 kallikrein-inhibiting U/mL of Trasylol/aprotinin, and 1 m*M* PMSF. This is a buffer for isolation of polypeptides expressed in bacteria from an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter *(4,5,7)*.

   a. Again, a base 10 buffer stock of Tris, NaCl, and EDTA can be prepared (this is stable indefinitely at 4˚C). Benzamidine is prepared as a 500X stock in water (it can be stored indefinitely at –20˚C). All other stocks are as for buffers 1 and 2.

   b. For bacterial preparations, we lyse the cells with lysozyme (from Sigma-Aldrich) prepared in buffer 5 as a 10X stock (10X is 10 mg/mL). Lysozyme is prepared freshly immediately before use and kept on ice until use. Discard unused portion.

   c. Lysozyme treatment is followed by degradation of the DNA with DNase, also prepared in buffer 5. We prepare 100X DNase-1 (Boehringer Mannheim) stock (10X is 2.5 mg/mL). DNase is prepared freshly immediately before use and kept on ice until use. Discard unused portion.

   d. An elution buffer containing 10X (0.5 *M*) glutathione is also prepared from this buffer. Be sure to readjust the pH of the glutathione buffer to 7.8—glutathione is extremely acidic. This should also be prepared immediately before use. Discard unused portion.

    e. We also prepare this buffer in 50% (v/v) glycerol, 0.1% (w/v) Triton X-100 instead of water. This is a storage buffer into which bacterially expressed proteins are dialyzed for storage in the freezer.

6. Buffer 6: 50 m*M* Tris-HCl (pH 8.0), 150 m*M* NaCl, 2.5 m*M* CaCl$_2$, 0.1% (w/v) Triton X-100, 0.1% (w/v) β-mercaptoethanol. This is a buffer for thrombin cleavage and removal of GST from bacterially expressed proteins *(4,5,7)*. We only cleave a few proteins (ERK1 and SAPK/JNK), all of which start as GST fusions, with this method. The remainder we elute with free glutathione (GSH) and use as GST fusions *(4,5,7)*. A 10X Tris, NaCl, CaCl$_2$ stock can be prepared and stored indefinitely at 4°C. Be sure to readjust the pH of the final buffer. The final buffer should be prepared freshly for each experiment.

7. Homemade enhanced chemiluminescence (ECL) reagents: This is far cheaper than any ECL kit on the market and it works just as well. Immunoblotting with this reagent is essentially the same as for any commercial kit that employs a horseradish peroxidase second antibody. At the end of the final washing after the second antibody, rinse the blot in PBS and drain completely. Immediately before developing the ECL blot, mix equal parts of solutions A and B and pour over the blot. Incubate for 60 s and expose as for standard ECL (*see* **Subheading 3.3.**).

    a. Base stocks:

        i. 100 m*M* Tris-HCl (pH 8.5); store at 4°C.

        ii. 30% H$_2$O$_2$ (available from Fisher); store at 4°C, and avoid contact with hands, eyes, or any chemicals prone to violent oxidation.

        iii. 250 m*M* luminol (5-amino-2,3-dihydro-1,4-phthalazinedione 3-aminophthalhydrazide) in water (Sigma-Aldrich); store at room temperature.

        iv. 90 m*M* p-coumaric acid in water (Sigma-Aldrich); store at room temperature.

    b. Solution A: 10 mL of 100 m*M* Tris stock, and 5.5 μL of H$_2$O$_2$ stock. Make no more than 1 h before use, store at 4°C until used.

    c. Solution B: 10 mL of 100 m*M* Tris stock, 100 μL of luminol stock, and 44 μL of p-coumaric acid stock. Make no more than 1 h before use; store at 4°C until used.

8. ATP/Mg

    a. Our kinase assays typically call for 50 μ*M* γ^{32}P-ATP/10 m*M* MgCl$_2$ *(4,5,7–9)*. We typically prepare a 5X (250 μ*M* γ^{32}P-ATP/50 m*M* MgCl$_2$) stock for use in assays. It is important to note that most commercial preparations of ^{32}P-ATP contain too little ATP to actually contribute significantly to the overall ATP concentration of the stock. This is especially true of some of the high specific activity products offered by New England Nuclear. Accordingly, unlabeled ATP must be used to adjust the concentration of ATP of one's 5X ATP stock to 250 μ*M* by using unlabeled ATP. The amount of γ^{32}P-ATP added will determine the specific activity.

    b. Measure the specific activity of the γ^{32}P-ATP by diluting the γ^{32}P-ATP stock 1000-fold and counting 5 μL by scintillation counting (Cerenkov counting is unreliable, especially in cold, dry climates prone to static electricity). In most instances we aim for between 1500 and 7000 cpm/pmol of ATP (we have provided counts here, not Becquerels/Curies, since the sensitivity/accuracy of scintillation counters varies). Higher specific activity allows for the detec-

tion of low-abundance phosphoproteins (i.e., autophosphorylation of kinases that are low in abundance). Lower activity (1500–2000 cpm/pmol) is, obviously, cheaper and is perfectly suitable for most simpler assays (such as SAPK/JNK or SEK1/MKK4 assays).

c. ^{32}P is a strong β-particle emitter. Accordingly, one should, of course, follow all institutional and governmental regulations for the handling of radioactivity and must receive appropriate training, as required by one's institution, before attempting to perform experiments with radioactive materials. In our laboratory, for safety purposes, it is assumed that once one opens the container of ^{32}P, everything one touches, along with one's work area, is automatically contaminated with radioactivity. Thus, we wear gloves (double gloves if using >2 mCi of radioactivity), lab coats, and closed-toe shoes (not sandals), and we perform all work on absorbent diaper paper with plastic backing. Also clean the pipet with a decontaminant (Count-Off from NEN is ideal) after use. Dispose of all consumables (pipet tips, tubes, absorbent paper, gloves) in the appropriate radioactive waste and scan the area with a Geiger counter, cleaning any surface contamination. All work should be done behind a Plexiglas shield at least 1-cm thick (2 cm if using particularly high specific activity ATP).

9. Laemmli sample buffer: 60 mM EDTA (pH 6.8), 40 mM DTT, 6% (w/v) sodium dodecyl sulfate (SDS), 30% (w/v) sucrose, 0.06 mg/mL of pyronin-Y (tracking dye—we prefer this to bromophenol blue because it has a more vivid color when diluted). This sample buffer contains EDTA, which will chelate the Mg in the kinase reaction. Accordingly, this buffer will stop the kinase reaction more rapidly than can SDS alone. The recipe is for a 6X stock. It can be diluted further with water as needed. Store in 2-mL aliquots at –80°C.

2.2. Recombinant Proteins

1. Proteins produced in bacteria:
   a. GST-c-Jun(1–135) (M_r = 45 kDa).
   b. GST-ATF2(2–89) (M_r = 39 kDa).
   c. SAPKα/JNK2 (M_r = 54 kDa if using p54 isoform or 46-kDa if using p46 isoform; **Table 3**).

   These proteins are all produced in bacteria as GST fusions from the pGEX-KG vector (*see* **Subheading 3.1.**) *(4,5,7,9)*. This vector has a small glycine "kinker" region between the thrombin cleavage site and the polylinker, which allows for more efficient thrombin cleavage. The SAPK/JNK is cleaved with thrombin while still immobilized on glutathione beads; however, the other polypeptides are eluted from the glutathione agarose with free glutathione. Methods for production of these proteins are in **Subheading 3.1.**

2. Proteins produced in transfected 293 cells:
   a. GST-SEK1/MKK4 (wildtype and K129R) (M_r = 68 kDa).
   b. GST-MKK6 (wildtype and K82R) (M_r = 68 kDa).

   These proteins are produced in 293 cells transfected with the cognate pEBG vector (*see* **Subheading 2.3.**) *(7,9)*. This vector employs the EF-1α promoter and the SV40 origin of replication, allowing for production of extremely large quantities

of GST-tagged protein from transfected 293 cells (up to 2–10 μg of protein/five 10-cm culture dishes) *(7)*. Methods for production of these proteins are provided in **Subheading 3.2.**

2.3. Plasmids

Our recombinant plasmids generally drive expression of epitope-tagged proteins, thereby allowing for efficient, selective immunoprecipitation of recombinant protein from transfected cells. We employ either the influenza hemagglutinin (HA, sequence: MYPYDFPDY), M2-FLAG (sequence: MDYKDDDDK), Myc 9-E10 (sequence: MEQKLISEEDL), or GST epitopes. Commercial antibodies to these epitopes (Sigma-Aldrich for anti FLAG, Santa Cruz Biotechnology for anti-Myc or anti-HA, Upstate Biotechnology for anti-GST) are widely available.

1. Vectors: We most frequently use the following vectors *(4,5,7,9)*:
   a. pMT3 (HA fusion vector driven by adenovirus major late promoter, Amp resistance).
   b. pCMV5-FLAG (FLAG fusion vector driven by cytomegalovirus [CMV] promoter, Amp resistance).
   c. pCMV-Myc (Myc fusion vector driven by CMV, Amp resistance).
   d. pEBG (GST fusions for mammalian expression, EF-1α promoter-driven, Amp resistance).
   e. pGEX-KG (GST fusions for bacterial expression, Amp resistance).
2. Protein-G-agarose (Pharmacia).
3. Glutathione agarose (Pharmacia).
4. Benzamidine agarose (Pharmacia or Sigma-Aldrich).
5. IPTG.
6. Thrombin (Sigma-Aldrich). Thrombin must be at least 3000 U/mg. Typically, an ~1 U/mL stock is made in water. This is snap frozen in liquid nitrogen (or dry ice–methanol) and stored at –80°C as 50-μL aliquots. It is stable at –80°C for 6 mo.

For studies of endogenous kinases, one may wish to obtain or purchase immunoprecipitating antibodies. Antibodies to many MAPKs, MEKs, and MAP3Ks are available commercially from any of the following vendors: Santa Cruz Biotechnology, Upstate Biotechnology, Promega (Madison, WI), Cell Signaling Technologies. We do not endorse any of these products and one should not infer that this list is inclusive or indicates the best sources. Commercial antibodies should be tested on a small scale under one's laboratory conditions. Many companies that make these reagents will offer trial samples for free, especially for new products. It is recommended that one try these before investing in the expense of commercial antibodies. Different experimental/animal cell systems each have their own quirks.

3. Methods

3.1. Preparation of GST-Tagged Proteins From Bacterial Extracts

This method is for proteins expressed from the pGEX-KG vector. These include GST-c-Jun(1–135), GST-ATF2(2–89), and GST-SAPK/JNK *(4,5,7,9)*.

3.1.1. Cell Growth and Induction

1. DNA for the desired clone can be transformed into appropriate host cells (JM109, DH5α, and so forth) using standard techniques. We find that JM109 often gives better protein yields. Select transformants with ampicillin. Isolate a single colony to prepare a glycerol stock.
2. Inoculate 20 mL of superbroth/50 μg/mL of ampicillin with a tiny scrape from the glycerol stock of the appropriate clone. Grow this to saturation and use it to inoculate 2 L of superbroth/50 μg/mL of ampicillin.
3. Grow at 37˚C until the OD_{590} is 0.5. Reduce the temperature of the culture to 25˚C (room temperature) and then add IPTG to 75 μg/mL. We find that room temperature incubation increases the yield of soluble protein. If doing this for the first time, we suggest doing a negative control grow-up, on a proportionally smaller scale, incubated without IPTG. In this manner IPTG induction and recovery of the protein can be monitored through the various steps without being confused by background proteins in the extract.
4. Incubate the culture overnight at 25˚C with good aeration (≥ 250 rpm in a rotary incubator).
5. For troubleshooting, *see* **Note 1**.

3.1.2. Lysis and Glutathione Agarose (GS-Agarose)

1. Prepare buffer 5 and lysozyme and DNase stocks in buffer 5.
2. Collect the bacteria by centrifugation (5000*g*).
3. Loosen the pellet from the bottom of the centrifuge bottle by vigorous vortexing/agitation. Make sure that there is no pellet material sticking to the bottom *before* adding lysis buffer; this will increase the exposure of the cells to the lysis buffer and increase yield. We prefer centrifuge bottles with conical bottoms because they allow for more efficient resuspension of the pellet.
4. Add 45 mL of buffer 5, to the pellet and resuspend well. Add 5 mL of the 10X lysozyme stock. Incubate on ice for 30 min. By 30 min the material should appear quite viscous owing to the presence of bacterial DNA in the extract.
5. Add 1 mL of the DNase stock and 1 *M* $MgCl_2$ to a final concentration of 2.5 m*M*. Incubate on ice with occasional swirling for 10 min. The material should now be somewhat less viscous. Be sure to take an aliquot of this material (2–5% of the total) to determine the total level of recombinant protein expression in the crude extract.
6. Spin the material at 100,000*g* for 30 min at 4˚C. Discard the pellet. Reserve an aliquot of the supernatant (the *same* percentage as in **step 5**). This will determine the proportion of expressed protein that is soluble and, therefore, usable.

7. During the spin, wash 1 mL (*settled*) of GSH-Sepharose (Pharmacia) with buffer 5. Leave the beads as a 1:1 suspension.

8. Add the beads to the supernatant and rotate at 4°C for 1 h. Alternatively, prepare a small disposable column and pass the extract over the column. If this is done, collect the flow-through and pass it back over the column an additional three times.

3.1.3. Elution With Free Glutathione

1. If choosing to elute the GST fusion protein with free glutathione, *do not* wash the beads into thrombin buffer (buffer 6). Instead, wash three times with buffer 5, three times with buffer 3 containing 1 *M* LiCl and three times with buffer 5. Leave the beads on the bottom of the tube with the buffer aspirated.

2. Prepare 0.5 *M* glutathione in buffer 5. Glutathione is extremely acidic, so adjust the pH back to 7.8 when the glutathione is dissolved.

3. Add buffer 5 to the beads to give a 1:1 suspension of beads and remove an aliquot of beads (the *same* percentage as in **Subheading 3.1.2., step 5**). This will be used to determine how much protein is bound to the beads. Then add 4 mL of buffer 5.

4. Add 10X glutathione-containing buffer 5 to the beads to give a final concentration of 50 m*M* glutathione and rotate at 4°C for 90 min. Collect the beads by centrifugation (1000*g*, 1 min) and reserve the supernatant. Repeat **step 3**, collecting the *same* percentage of beads. This will show you how much material was not eluted. Take the same percentage of the supernatant as well, to show how much protein was eluted. Assay the protein in the supernatant to determine the concentration of protein eluted.

5. Dialyze the material into buffer 5 prepared with 50% (v/v) glycerol/0.1% (w/v) Triton X-100, and store the material at –20°C. It can be kept for at least 6 mo.

6. Run a gel on all the reserved aliquots to assess the progress of the purification.

3.1.4. Thrombin Cleavage

Be sure that the protein of interest has no internal thrombin cleavage sites (GR/G, GR/P, or PR/G; the slash indicates the site of cleavage—we find that only SAPK/JNK can be reliably recovered using this procedure). *Do not* cleave the Jun or ATF2 with thrombin. Elute these with free glutathione *(5,7)*. Make sure that the cleaved product is sufficiently large to run reliably on an SDS gel (>12,000 Daltons).

1. Wash the beads with 5 × 10 mL washes of buffer 6. Add buffer 6 to give a 1:1 suspension of beads and buffer. Remove an aliquot (the same percentage as in **Subheading 3.1.2., step 5**). This will provide a measure of the amount of recombinant protein bound to the beads. Add sufficient buffer 6 to bring the volume of the slurry to 4 mL.

2. Add thrombin to the bead suspension to give 20 U/mL.

3. Rotate for 1 h at room temperature. Spin out the beads and keep the supernatant. Respin the supernatant to ensure removal of all beads. Take an aliquot of the

supernatant (the same percentage as in **step 1**) to give you an estimate of the recovery of cleaved material. Resuspend the beads as a 1:1 suspension and take an aliquot of these (the same percentage as in **step 1**) to determine how much material was not cleaved and how much of the cleaved material stuck to the beads nonspecifically. Assay the protein in the supernatant (we use the Bio-Rad reagent).

5. Take the supernatant from **step 3** and remove the thrombin by rotating for 1 h at 4°C with 1.5 mL of benzamidine Sepharose (settled beads) equilibrated in thrombin cleavage buffer. Recover the beads by centrifugation (3000g, 1 min) and keep the supernatant. Add EDTA to the supernatant to a final concentration of 5 mM. Take an aliquot (same percentage as in **step 1**) to measure recovery of the cleaved protein.

6. Dialyze the supernatant for at least 4 h as described in **part C**. Store the protein at –20°C. Material prepared in this manner is stable for 3–6 mo.

3.2. Purification of Proteins Expressed in 293 Cells From the pEBG Vector

We use this method for preparation of *soluble* GST-tagged polypeptides that cannot be purified from bacterial extracts owing to intractable problems with stability, solubility, and so forth. It is especially good for larger proteins. Examples include SEK1/MKK4, MKK3, MKK6, p38, MEKK1, ASK1, TRAFs-2 and -6, and the mammalian Ste20 homolog germinal center kinase (GCK). Constructs should be subcloned into the pEBG vector. Yields are generally good (2–20 µg/preparation), and stable, highly purified preparations can be obtained. Proteins prepared in this manner are not immobilized but, instead, are free in solution, and are thus amenable to more sophisticated analyses such as kinetics or calorimetry.

Should one wish to prepare small-scale analytical quantities of immobilized GST-tagged proteins expressed in transfected cells, use the method described in **Subheading 3.3.** For troubleshooting and more information, *see* **Note 2**.

1. Transfect 293 cells with the desired pEBG construct. Typically, we use Lipofectamine (Gibco-BRL) according to the manufacturer's instructions. This method is expensive, however, and should this be a problem, 293 cells can be transfected easily using the standard CaPO$_4$ method.

2. One to 2 d later, harvest the cells. The number of cells used and the timing of the harvest (1 or 2 d posttransfection) depend on the toxicity of the construct. For proteins involved in the stress response, we favor harvesting at 16–24 h posttransfection; otherwise, cells expressing the toxic proteins undergo accelerated death, decreasing the yield and effective transfection efficiency. Proteins for which this is particularly important include the following: ASK1, MEKK1, GCK, TRAF2, any protein with a death domain, and constitutively active mutants of SEK1/MKK4, MKK3, MKK6, and MKK7.

   Remember, even if genetic or biologic studies indicate that the protein being used is not proapoptotic, overexpression of the protein—as is being done in this protocol as a means of producing polypeptide for in vitro studies—may

nonspecifically lead to apoptosis. Of course, it is always wisest to spend some time examining empirically the kinetics of expression so as to determine, in one's hands, the point at which maximum expression reliably occurs (*see* **Note 2**).

In most cases, for production of sufficient quantities of protein, we recommend transfection with 5 µg of plasmid. We recommend, however, that one determine ahead of time the amount of plasmid that, in one's hands, will provide maximal expression. Regarding how many plates to transfect, usually 10–20 10-cm plates are enough, but, 20–40 plates are necessary for toxic/proapoptotic proteins; however, the only limitations are one's tolerance for tissue culture and one's budget for serum. If one wishes to activate kinases *in situ* with natural ligands, one should use the appropriate stimuli (**Table 1**).

3. Using a dounce homogenizer, lyse the cells in buffer 2. If one needs to preserve protein Ser/Thr-phosphorylation, include 50 m*M* β-glycerophosphate in buffer 2. Add the PMSF immediately before harvesting the cells. One milliliter of lysis buffer per 10-cm plate is usually enough. However, if more than ten 10-cm plates expressing a single protein are being harvested, we recommend pooling the lysate into a volume no greater than 10 mL.

4. Spin out the unbroken cells, nuclei, and membranes (100,000*g*, 40 min). Discard the pellets. Add Triton X-100 to 0.1% (w/v). If Tyr phosphorylation needs to be preserved, add Na_3VO_4 to 0.1 m*M*.

5. Equilibrate a minicolumn of 150 µL of (settled) glutathione agarose/10 mL of extract with buffer 2/Triton X-100/vanadate. Pass the material over the column, collect the flow-through, and reapply the flow-through to the column a total of four times.

6. Wash the beads three times using 5-mL washes with low salt buffer 3. Wash the beads three times using 5-mL washes with buffer 3 containing 50 m*M*–1 *M* NaCl/ LiCl. The amount of salt used will depend on the desired stringency of the washes—low salt will more likely preserve protein-protein interactions based on ionic interactions. High salt will remove undesirable contaminants based on ionic interactions. Finally, wash three times using 5-mL washes with low-salt buffer 3.

7. If desired, elute into low salt buffer 3 with 50 m*M* glutathione (free acid—*be sure* to readjust the pH of the elution buffer after preparing it, since glutathione is extremely acidic) by tumbling for 1 h at 4°C. Otherwise, keep the protein on beads and wash into 50% glycerol-containing low salt buffer and store at –20°C.

8. For eluted proteins, discard the beads by centrifugation (3000*g*, 1 min) and dialyze the supernatants into buffer 2 prepared with glycerol (50% [v/v]), 0.1% Triton X-100, and without sucrose. Store at –20°C. We find that GST-tagged mammalian proteins are stable for up to 3 mo if stored properly.

3.3. Basic Procedure for Immunoprecipitation/Isolation of Protein Kinases

What follows is a basic protocol for immunoprecipitation of protein kinases for immunoprecipitation (IP)-kinase assay *(4,5,7,9)*. Because antibody titers vary considerably, it is important to determine empirically how much antibody is needed to see a reliable signal. Here are some useful guidelines: For antibodies

in hybridoma supernatants, dilute 1:10–1:20 (50–100 µL/mL of lysate); For commercial anti-FLAG, Myc, or HA, dilute 1:200–1:1000 (1–5 µL/mL of lysate).

If one obtains a "homemade" antibody from a collaborator, be sure to ask him/her how much to dilute the antibody in order to detect reliably the enzyme of interest when starting with extracts of the cells of interest.

For GST-tagged proteins to be analyzed as immobilized complexes, we generally use 20 µL of (settled) beads/mL of cell lysate.

1. Transfect the cells, as desired, with the appropriate plasmid(s). As with preparation of GST-tagged proteins in **Subheading 3.2.**, the amount of plasmid and time of transfection vary. For studies in which one wishes to examine activation of a protein kinase by a potential upstream regulator, we suggest expressing *less* of the effector kinase that is to be assayed and more of the upstream activator. This is especially an issue if the kinase being examining has a high basal activity (*see* **Subheading 3.4.**). Treat cells with the desired stimulus (**Table 1**). For treatment of cells with a stimulus whose characteristics are not known, we highly recommend that a time course and dose response first be performed. Kinetics and dose responsiveness often vary considerably among different cell types and stimuli, and the use of a single dose or time may result in an incomplete picture of the regulation of a particular enzyme in vivo. Use a wide time span (30 s to 90 min) and a broad range of doses (as a rule, if doses are multiplied by a factor of 3, e.g., 0.1, 0.3, 1, 3, 10, 30, and 100 ng/mL of TNF, for example, three orders of magnitude can be covered with relatively few doses).

2. Lyse the cells in ice-cold buffer 1. Allow the cells to solubilize for 5 min on ice.

3. Spin out the unbroken cells and nuclei, and normalize the protein in the supernatants such that the concentration of protein is equal to that of the sample that originally had the lowest protein concentration. We use the Bradford assay, and measure 10–20 µL of sample. Be sure to save ~100 µL of normalized extract for Western blotting if proteins from transfected cells are being assayed. To 1 mL of extract supernatant, add the appropriate amount of antibody (see above). Then add enough protein-G Sepharose to give 20 µL of (settled) beads/mL of lysate. If GST-tagged proteins are being isolated, add 20 µL of (settled) glutathione agarose/mL of cell lysate.

4. Rotate for 3 h at 4°C. Wash three times in buffer 1, three times in buffer 3 (whether or not a large amount of salt is included should be determined empirically), and three times in buffer 4. Leave the beads as a 1:1 suspension in buffer 4.

5. If assaying immobilized GST-tagged kinases, add free glutathione (adjusted to pH 7.2) to a final concentration of 20 m*M*. This will prevent binding of GST-tagged substrates (e.g., GST-c-Jun) to the beads; this binding lowers the efficiency of the kinase reaction.

3.4. Simple Assay of Immobilized MEKs (SEK1/MKK4)

This protocol is quite adaptable. We have used this method to assay immunoprecipitated SEK1/MKK4 and MKK7 (with SAPK/JNK as substrate), MKKs-3 and -6 (with p38 as substrate), and MEK1 (ERK1 as substrate) *(7,9)*.

The assay measures phosphorylation of the relevant MAPK polypeptide and does not subsequently measure activation of the MAPK. An assay for that is provided in **Subheading 3.5.** The assay is amenable to use with immunoprecipitates or GST pulldowns. For troubleshooting and more information, *see* **Notes 3** and **4**.

We present this assay for immunoprecipitated SEK1/MKK4; however, the basic procedure is suitable for assay of soluble SEK1/MKK4 (or other MEKs) isolated, such as, by column chromatography *(4)*. In this instance, we recommend that 40 µL of SEK1/MKK4 preparation be substituted for the 40 µL of SEK1/MKK4 immunoprecipitate. Typically, the soluble SEK1/MKK4 should be diluted in buffer 4. The degree of dilution must be determined empirically such that an assay delivers linear incorporation of phosphate into SAPKα/ JNK2-K55R over at least 20 min.

The following reagents will be needed (to determine how much is needed, multiply the amounts indicated in the assay by the number of assays to be performed). As discussed in **Subheading 2.1.**, be sure to follow all precautions and regulations regarding the handling of radioactivity.

    a. Immunoprecipitated or glutathione agarose-immobilized SEK1/MKK4 (*see* **Subheading 3.3.**).

    b. SAPKα/JNK2-K55R prepared as in **Subheading 3.1.** This is a kinase-dead mutant of SAPK/JNK. Dilute the SAPK/JNK to 300 µg/mL in buffer 4. One may also wish to perform parallel assays on SAPK/JNK phosphoacceptor site mutants (T183A/Y185F/K55R) as a means of establishing more rigorously that the observed phosphorylation is indeed MEK catalyzed. These mutants should not be phosphorylated under conditions including K55R.

    c. γ^{32}P-ATP-Mg stock (250 µM γ^{32}P-ATP/50 mM MgCl$_2$) prepared as in **Subheading 2.1.**

Then follow these steps:

1. Preheat a water bath or a Thermomixer (we use the Eppendorf model 5436) to 30˚C.
2. To 40 µL of slurry containing 20 µL of settled SEK1/MKK4 beads, add 20 µL of diluted SAPK/JNK.
3. Place the tubes in the water bath or the Thermomixer.
4. Start the timer (we recommend counting up from 0, rather than down from 15 min), add 15 µL of 5X ATP stock to each tube in 15- to 20-s increments (if it is difficult to add this fast, slow down to a comfortable speed). When all the tubes have ATP, start the Thermomixer. If using a conventional water bath, vortex the samples every 20 s.
5. When the timer reads 15 min, add 25 µL of 4X Laemmli sample buffer to the first tube that received ATP/Mg. Sequentially to the subsequent tubes, add sample buffer in the same time increments used to add the ATP.

6. Heat the samples to 80°C for 5 min. Cool and resolve proteins by SDS-polyacrylamide gel electrophoresis (PAGE) (we recommend a 10% high-bisacrylamde or a 15% low-bisacrylamide gel).

7. After SDS-PAGE, remove the dye front from the gel. This will significantly reduce background radioactivity in the gel. At this point there are two options: The gel can be stained/destained (Coomassie blue is adequate), dried, and subjected to autoradiography or phosphoimaging, or, alternatively, proteins can be transferred to Immobilon (polyvinylidene difluoride) membranes, stained, and destained (Coomassie blue is adequate). The latter method, while somewhat more costly, shortens the time needed to reduce the background radiation to an acceptable level and allows immunoblotting the immunoprecipitates directly as a means of detecting expressed proteins. Bear in mind, however, that if one or more of the proteins of interest migrates at or near the IgG light or heavy chains, it will be impossible to use the immunoprecipitates to immunoblot for expression of the protein. Instead, use the reserved normalized crude cell extract (*see* **Subheading 3.3.**) for immunoblotting.

8. Locate the radioactive bands by autoradiography or phosphoimaging. Excise the appropriate bands from the gel and subject to scintillation counting. The amount of phosphate incorporated into the band can be calculated according to the following formula:

pmol of phosphate incorporated/min = [cpm in band/specific activity of ATP (in cpm/pmol ATP)]/15 min

The assay can also be quantitated, albeit less accurately, in the phosphoimager by spotting 1 μL of a 1:250 dilution of ATP/Mg stock (this gives 1 pmol) onto a piece of Immobilon and measuring the phosphorimager signal of the spot. Under these circumstances, the amount of phosphate incorporated can be calculated according to the following formula:

pmol of phosphate incorporated/min = (phosphoimager counts in band/phosphoimager counts in ATP calibration spot)/15 min

9. If you are assaying SEK1/MKK4 from several parallel transfections, it will be necessary to immunoblot to show that changes in SEK1/MKK4 activity are not accompanied by changes in SEK1/MKK4 expression levels, and are in fact the result of the experimental manipulations under investigation. SEK1/MKK4 and, indeed all of the MEKs are not easily detected on immunoblots of immunoprecipitates. We recommend a parallel immunoblot of the crude extracts set aside prior to immunoprecipitation. As described in **Subheading 3.3.**, the amount of antibody used in an immunoblot differs depending on the source and type of antibody and should probably be determined empirically. We use ECL as the detection method; and most laboratories have their own procedures for this. These methods are, in general, suitable for the types of kinases described herein. We present a brief description highlighting some of our approaches. Stain the blot

with Coomassie Blue. Because the stain will gradually dissipate during the procedure, use a syringe needle to poke holes at the migration positions of the molecular weight marker proteins, and to mark the top center of each lane. We block immunoblots with phosphate-buffered saline (PBS)/0.5% (v/v) Tween–20/1% (w/v) Triton X-100, 5% (w/v) nonfat dry milk. All incubations with antibody and second antibody are performed in this blocking buffer, as are all washes, up until the ECL reaction. Immediately prior to ECL, rinse the blot in regular PBS twice and drain any remaining buffer. Mix ECL solutions A and B (*see* **Subheading 2.1.**) and add to the blot for 1 min. Expose to X-ray film.

3.5. Complete Assay of Immobilized MEKs (SEK1/MKK4)

This assay measures the ability of SEK1/MKK4 to activate SAPK/JNK's c-Jun kinase activity. Several control assays will need to be added, specifically an assay of the basal c-Jun kinase activity of the SAPK/JNK preparation and an assay of the residual c-Jun kinase activity in the SEK1/MKK4 immunoprecipitate. Again the basic structure of this assay is adaptable and we have used it for the assay of several MEKs *(4,7,9)*.

We present this assay for immunoprecipitated SEK1/MKK4; however, the basic procedure is suitable for assay of soluble SEK1/MKK4 (or other MEKs) isolated such as by column chromatography *(4)*. In this instance, we recommend that 40 μL of SEK1/MKK4 preparation be substituted for the 40 μL of SEK1/MKK4 immunoprecipitate. Typically, the soluble SEK1/MKK4 should be diluted in buffer 4. The degree of dilution must be determined empirically such that an assay delivers linear incorporation of phosphate into c-Jun over at least 20 min.

The same reagents as for the simple assay in **Subheading 3.4.** will be needed with the following modifications: Prepare a "blank" immunoprecipitate by carrying out the immunoprecipitation procedure using buffer instead of cell lysate. This will serve as the control for basal SAPK/JNK activity. A sample of the buffer in which the SAPK/JNK is stored (generally buffer 2 prepared in 50% [v/v] glycerol/0.1% [w/v] Triton X-100) will also be needed to serve as a negative control for residual c-Jun kinase activity in the SEK1/MKK4. SAPK/JNK should be wild-type, not kinase-dead K55R. Also needed is GST-c-Jun(1–135) prepared as in **Subheading 3.1.** and diluted to 200 μg/mL in buffer 4. Again, the amounts of each material needed will be the product of the amounts listed per assay and the number of assays to be performed.

1. Proceed with the assay in a manner identical to the simple assay in **Subheading 3.4.** with the following modifications: The wild-type SAPK/JNK should be diluted in buffer 4 to a concentration of 40 μg/mL. In an identical manner, for "control B," dilute the buffer in which the SAPK/JNK is stored into buffer 4.

  a. Include the following control samples:
   i. Control A: Blank immunoprecipitate plus SAPK/JNK (no SEK1/MKK4). This will measure the basal activity of the SAPK/JNK.
   ii. Control B: SEK1/MKK4 immunoprecipitate plus buffer (no SAPK/JNK). This will measure any contaminating Jun kinase activity in the SEK1/MKK4 preparation.
  b. On completion of the incubation with SAPK/JNK, do not add Laemmli sample buffer. Instead, add in the same time increments at which the ATP was added 15 μL of the c-Jun and 10 μL more of ATP/Mg mix.
 2. Allow the reaction to proceed for an additional 15 min at 30°C. Stop the reaction with 33 μL of Laemmli sample buffer in the timed manner described in **step 1b**.
 3. SDS-PAGE, immunoblotting, and autoradiography are the same as for **Subheading 3.4.** In this instance, however, quantitate incorporation of phosphate into the GST-c-Jun. The quantitation of phosphate incorporation is the same as in **Subheading 3.4.** To calculate true SEK1/MKK4 activity, use the following formula:

(phosphate incorporated/min into c-Jun in presence of both SEK1/MKK4 and SAPK/JNK) — [phosphate incorporated/min into c-Jun in presence of SEK1/MKK4 only (Control B) + phosphate incorporated/min into c-Jun in presence of SAPK/JNK only (Control A)]

3.6. Simple Assay for Immobilized MAP3Ks (MEKK1)

 This assay measures the MEKK1-catalyzed phosphorylation of kinase-dead SEK1-(K129R). It does not actually quantitate activation of the MEK *per se*. An assay for that is in **Subheading 3.7.** The basic structure of this assay is similar to that of the simple MEK assay described in **Subheading 3.3.** The method is also adaptable to the assay of most other stress-activated MAP3Ks *(9)*. We have used this method to assay the MLKs, ASK1, as well as MEKKs-3 and -4 ([**refs. 9** and **10**]; unpublished).

 Immunoprecipitated/immobilized MAP3K (MEKK1) is required for the assay. We highly recommend that a full-length construct be used for all assays of stress-activated MAP3Ks, including MEKK1, that do not involve explorations of the effects of truncation/deletion. Even full-length MEKK1 has a high basal activity and quantitation of activation is difficult. This is owing in large part to spontaneous in vivo oligomerization-dependent activation, and to spontaneous activation in the immune precipitate during the assay. Accordingly, if recombinant (transfected) MEKK1 is being examined, we suggest that a very small amount per dish be transfected (<50 ng plasmid/10-cm dish for a plasmid with a strong promoter such as CMV). Alternatively, one can subclone into a vector with a weaker promoter or prepare stable cell lines with an inducible promoter. As with SEK1/MKK4, we recommend that extracellular stimuli be

tested as MAP3K (MEKK1) agonists in time course and dose-dependence assays in order to determine rigorously whether the agonist in question can activate MEKK1.

The assay also requires GST-SEK1-(K129R) prepared from transfected 293 cells expressing the pEBG construct (*see* **Subheading 3.2.**). Dilute to 20 μg/ mL in buffer 4. To prepare this SEK1, *do not* stimulate the cells with any agonist. Ideally, the SEK1/MKK4 should be in a "resting" state and not phosphorylated at the regulatory phosphoacceptor sites (S257, T261) *(1)*.

Because of the high spontaneous activity of MEKK1 and other stress-activated MAP3Ks, a low-concentration 5X ATP stock (50 μ*M* γ^{32}P-ATP/50 m*M* MgCl$_2$ to give a final ATP concentration of 10 μ*M* in the assay) should be prepared. We find that this helps reduce the high basal activity of MEKK1 and other stress-activated MAP3Ks.

1. Preheat a water bath or Thermomixer to 30°C.
2. To 40 μL of slurry containing 20 μL of settled MEKK1 immunoprecipitate, add 20 μL of diluted SEK1-(K129R). Place the tubes in the water bath or Thermomixer.
3. As in **Subheading 3.4.**, start the timer and add 15 μL of the low-concentration ATP/Mg mix to each tube in comfortable, but regularly timed, increments. The actual duration of the assay depends on the experimental circumstances. Thus, MEKK1 and other stress-regulated MAP3Ks tend to autoactivate not only in vivo on overexpression, but during IP kinase assays. Accordingly, if one wishes to measure activation of MEKK1, not only should a minimal quantity of MEKK1 (or assay the endogenous enzyme) be transfected, as described above, but the duration of the assay should be shorter. We recommend a 2-min assay as the best way to detect activation *in situ* of MEKK1.
4. When the assay time is completed, starting at the first tube, add 25 μL of 3X Laemmli sample buffer in the same timed increments at which the ATP/Mg mix was added.
5. Proceed as in **Subheading 3.4.** for SDS-PAGE autoradiography; assay quantitation; and, as necessary, immunoblotting.

3.7. Complete Assay of Immobilized MAP3Ks (MEKK1)

This three-tiered assay measures MEKK1-catalyzed activation of SEK1/ MKK4's ability to activate SAPK/JNK. The approach can be used to measure all MAP3Ks. The assay was developed from our original assay for Raf-1 activation of MEK1 *(4)*. The three-tiered assay is not ideal for determining MAP3K regulation by upstream stimuli, because of the telegraphic nature of the assay and the resulting completely nonlinear kinetics of the results. However, the assay is the "gold standard" for determining whether a particular kinase possesses MAP3K activity. For this reason, we include the MEKK1 version of the assay. The following reagents will be needed:

a. Immunoprecipitated MEKK1 as in **Subheading 3.6.**
b. GST-SEK1/MKK4 (wild type, not kinase dead) purified from transfected 293 cells (*see* **Subheading 3.2.**). To prepare this SEK1, *do not* stimulate the cells with any agonist. Ideally, the SEK1/MKK4 should be in a "resting" state and not phosphorylated at the regulatory phosphoacceptor sites (S257, T261) *(1)*. Dilute to 5 μg/mL in buffer 4.
c. SAPKα/JNK2 (wild type) and GST-c-Jun-(1–135) purified from bacterial extracts (*see* **Subheading 3.2.**). Dilute the SAPK/JNK to 20 μg/mL and the GST-c-Jun to 500 μg/mL.

Prepare a duplicate set of assay tubes for each assay point. For control assays, a "blank" immunoprecipitate prepared from buffer instead of cell lysate, as well as buffer in which the SEK1/MKK4 and SAPK/JNK are diluted will be needed. A portion of this buffer should be diluted into buffer 4 in an identical manner to the dilution of the SEK1/MKK4. The remainder should be diluted as for the SAPK/JNK. These will be used for the following controls:

a. Control A: blank immunoprecipitate plus SAPK/JNK, no SEK1/MKK4—measures Jun kinase contributed by SAPK alone.
b. Control B: blank immunoprecipitate plus SEK1/MKK4 (no SAPK/JNK)—measures contaminating Jun kinase contributed by SEK1 alone.
c. Control C: MEKK1 immunoprecipitate alone (no SEK1/MKK4 or SAPK/JNK)—measures contaminating Jun kinase contributed by MEKK1 alone.
d. Control D: MEKK1 immunoprecipitate plus SEK1/MKK4 (no SAPK/JNK)—measures contaminating Jun kinase contributed by both MEKK1 and SEK1/MKK4 in the absence of added SAPK/JNK.
e. Control E: MEKK1 immunoprecipitate plus SAPK/JNK (no SEK1/MKK4)—measures Jun kinase contributed by MEKK1 plus SAPK/JNK in the absence of added SEK1/MKK4.

1. Preheat a water bath or Thermixer to 30˚C.
2. To a 40-μL of slurry containing 20 μL of settled MEKK1 or blank beads, add 20 μL of buffer or diluted GST-SEK1. Place the tubes in a water bath or Thermomixer. Add 15 μL of the standard 5X ATP/Mg mix (*see* **Subheading 2.1.**) in comfortable, regularly timed intervals and proceed with the reaction as in **Subheading 3.4.**
3. After 20 min, centrifuge the samples for 10 s at 3000*g* in a microfuge. Starting at the first tube and proceeding at the same timed intervals at which the ATP was added, remove 40 μL of the supernatant and place in cognate duplicate tubes. Put the duplicate tubes with the supernatant in the water bath or Thermomixer. Discard the remaining supernatants and beads. This step removes the MEKK1 and strikingly reduces background Jun kinase activity.
4. Start the timer. Starting at the first tube, and proceeding in comfortable, regularly timed intervals, add 20 μL of diluted SAPK/JNK to the 40 μL of supernatant.
5. Quickly reset the timer to zero and, starting at the first tube, and proceeding in comfortable, regularly timed intervals, add 5 μL more of the standard ATP/Mg mix.

6. After 20 min, starting at the first tube, and proceeding in comfortable, regularly timed intervals, add 10 μL of diluted GST-c-Jun to each tube, and then repeat **step 5**.

7. After 15 min, starting at the first tube, and proceeding in comfortable, regularly timed intervals, stop the reaction with 16 μL of 6X Laemmli sample buffer.

8. Proceed with SDS-PAGE and autoradiography/phosphoimaging as in **Subheading 3.4.** Quantitate phosphate incorporated into c-Jun as in **Subheading 3.4.** To calculate true MEKK1 activity use the following formulas (activity indicates phosphate incorporated into c-Jun):

$$\text{SEK1/MKK4 basal activity} = \text{activity in Control B} - \text{Control A}$$

"Gross" MEKK1 activity = [(activity in sample containing MEKK1, SEK1/MKK4 and SAPK/JNK) – (Ccontrol D + Control E)] + Control C

Stimulated SEK1/MKK4 activity = Gross MEKK1 activity – basal SEK1/MKK4 activity

4. Notes

1. The bacterial GST-tagged protein does not express/purify properly: In most instances this is owing to *in situ* denaturation and relegation of the recombinant protein to the inclusion body. Obviously, if this is an absolutely intractable problem, one can switch to expression in 293 cells from the pEBG vector. Enough material will still be obtained to do the assays described. Here are additional suggestions:

   a. Reduce the growing time in IPTG. Often, as bacteria accumulate more and more recombinant protein the protein will end up in the inclusion body.

   b. Reduce IPTG. This will slow the rate of protein production and allow for better folding of the recombinant protein, preventing degradation and/or movement to the inclusion body.

   c. Increase IPTG. It is possible that suboptimal IPTG is being used and more will lead to greater protein production.

   d. Always express bacterial proteins at room temperature (20–25°C). As with reduced IPTG, this reduces the rate of protein synthesis and allows for slower, more productive protein folding.

   e. Use a bacterial strain such as BL-21 (DE3) pLysS, which has reduced protease activity.

   f. Always be sure to provide good aeration (≥250 Rpm) and adequate nutrients (superbroth).

   g. Avoid denaturation/renaturation procedures such as urea or guanidinium followed by step dialysis, and *never* use a sonicator to lyse cells. These methods drastically reduce recoverable amounts of protein kinases and substrates.

   h. If the bacteria do not lyse after treatment with lysozyme (this is a problem with some constructs), there is an additional trick to opening the cells. This method works in many cases and does not harm the proteins on which we have tried it (ATF2, in particular), but is a violation of every shibboleth of protein biochemistry. Add 10 *M* NaOH dropwise until the cells lyse (the solu-

tion becomes viscous). *Immediately* add 5 mL of the 10X buffer 5 base stock (adjusted to pH 7.8). Then q.s. to 50 mL with 1X buffer 5.

2. The recombinant protein fails to express in mammalian cells: This is a frequently encountered problem when recombinant constructs encoding proapoptotic or unstable proteins are expressed in mammalian cells. Some potential solutions include the following:

    a. Express the protein for less time. Most conventional transfection protocols suggest waiting for 48 h or longer after the initial transfection procedure for harvesting. We have even encountered procedures in which transfected cells are split, with the goal being to expand the number of cells expressing a given construct (and, it is hoped, the yield) from a high-copy-number plasmid. The problem is that toxic proteins will often kill the cells during this time, lowering yields even for those investigators most accomplished at transfection. We find that most plasmids reach a maximal expression level at 12–16 h (e.g., overnight) posttransfection. So why not just harvest after an overnight incubation? Better yet, use immunoblotting to determine empirically the time course of expression of the construct.

    b. A second problem can arise if two constructs are coexpressed and one drives down the expression of the other. We have encountered this problem, on occasion, when coexpressing SAPK/JNK, or SEK1/MKK4 with MEKK1. Again, this was owing to a nonspecific toxic effect of the MEKK1, and the problem can be significantly overcome by reducing transfection time. If one is trying to assess the effects of protein B on protein A and B drives down the expression of A (thus making the expression of A in cells transfected with A alone greater than that in cells expressing both B and A), it is often necessary to vary empirically the levels of each protein such that balanced expression of the protein(s) of interest (A) is achieved.

    c. It is conceivable that some kinases are regulated by proteolysis. Indeed, cyclin proteolysis is a hallmark of cell-cycle regulation, and there is some evidence that MEKK1 is cleaved into a constitutively active fragment under certain conditions *(1)*. We have generally not observed regulation of MEKs or MAPKs by proteolysis. Of course, if degradation in response to the conditions being tested is consistently observed, this may be an avenue for further research. If, however, the degradation is attributed to the overexpression of the protein (e.g., the endogenous protein never behaves in this manner), or to the vector system employed (more of an issue with strong promoters), one can switch to weaker promoters or employ caspase inhibitors (DEVD, ZVAD) or proteasome inhibitors (lactacystin or MG132). Be careful with the use of proteasome inhibitors, however, because these can, on prolonged incubation (>6 h), trigger apoptosis. Moreover, use of these inhibitors may mask a biologic process that compels further investigation.

3. The kinase being assayed has excessive basal activity and regulation by upstream stimuli cannot be measured: This problem occurs most frequently for stress-activated MAP3Ks. These enzymes are activated in part by oligomerization and autophosphorylation *(1,9)*. Accordingly, overexpression and immunoprecipita-

tion can artificially prompt oligomerization, while addition of ATP can trigger autophosphorylation. We have also occasionally seen high basal activity in assays of overexpressed wild-type stress-activated MEKs, notably MKK6 and MKK7. The reasons for MEK activation are unclear but may be owing to an overwhelming of the available pool of phosphatases, a situation that would allow for activation of the MEKs at basal rates during the period of expression. The following suggestions can alleviate these problems:

a. Transfect a minimal amount of the recombinant expression construct (5–100 ng of plasmid/10-cm plate of cells is a good range). This will reduce basal in vivo activation of the kinase.

b. For MAP3K constructs/assays, reduce the ATP concentration as described in **Subheading 3.6.** An ATP final concentration of 10 μM is a good start.

c. For MAP3K constructs/assays, shorten the duration of the assay. Try assaying for 2 min or less.

d. Use expression vectors that have weaker promoters or are inducible.

4. The kinase being assayed is inactive. This may be owing to one of two possibilities: First, the stimulus/activator used in an attempt to activate the kinase in vivo or in vitro is not a physiologically relevant activator of the kinase in the cellular or biochemical context under investigation. This may be a cell-type problem, or a genuine negative result. It is always ideal, when possible, to include a positive control for these sorts of assays. For early tests of the stimuli that recruited SAPK/ JNK, we used cycloheximide *(6,8)* as a barometer of SAPK/JNK activation. In that way we knew our conditions for detecting activation of the SAPK/JNK were suitable. Similarly, for assays of MLK3 activation of SEK1/MKK4, we ran parallel assays of MEKK1 activation of SEK1/MKK4, thus ensuring that we could detect activation of SEK1/MKK4 in vitro *(10)*. Second, it is possible that conditions in the assay or in the stored enzyme(s) are reducing activity. In this casee, the likeliest culprits are contaminating Ser/Thr phosphatases, proteases, and/or bacteria. Ser/Thr phosphatases can be further reduced by adding additional Ser/ Thr phosphatase inhibitors such as 200 nM okadaic acid, 200 nM microcystin, and/or 200 nM calyculin to the stored kinase.

a. If proteolysis is a persistent problem (e.g., what little purified material recovered appears as a ladder of unexpectedly lower molecular weight bands on SDS-PAGE), as a last resort diisopropyl fluorophosphate (DFP) can be employed as a protease inhibitor. This is an extremely toxic chemical, however, and many institutions ban it altogether, or require extreme safety measures for use. Check with the institutional safety officer before proceeding. At a minimum, if DFP is handled, place all objects that come in contact with DFP into 1 M NaOH for 20 min. Perform all manipulations of DFP in a fume hood and wear double gloves. Like PMSF, DFP is labile in water. After ~1 h, it is mostly degraded. However, there are still toxic levels in solutions even after an overnight incubation.

b. Bacteria can be thwarted with 0.01% (w/v) NaN_3 (also toxic). Alternatively, some kinases, including SEK1/MKK4, can be aliquoted into small lots (<100 μL),

snap frozen in liquid nitrogen, and stored at –80˚C, which will more effectively reduce bacterial contamination. We find, however, that most recombinant signaling proteins (MKK6, p38, SAPK/JNK, ERK, as well as substrates) are best stored at –20˚C in 50% glycerol buffers as described. Sterile filtration is not a good option because this often leads to unavoidable loss of the protein on the filter.

References

1. Kyriakis, J. M. and Avruch, J. (2001) Mammalian mitogen-activated protein kinase pathways activated by stress and inflammation. *Physiol. Rev*. **81,** 807–869.
2. Tamura, K., Sudo, T., Senftleben, U., Dadak, A., Johnson, R., and Karin, M. (2000) Requirement for p38α in erythropoietin expression: a role for stress kinases in erythropoiesis. *Cell* **102,** 221–231.
3. Adams, R. H., Porras, A., Alonso, G., et al. (2000) Essential role of p38α MAP kinase in placental but not embryonic cardiovascular development. *Mol. Cell* **6,** 109–116.
4. Kyriakis, J. M., App, H., Zhang, X.-F., et al. (1992) Raf-1 activates MAP kinase–kinase. *Nature* **358,** 417–421.
5. Kyriakis, J. M., Banerjee, P., Nikolakaki, E., et al. (1994) The stress-activated protein kinase subfamily of c-Jun kinases. *Nature* **369,** 156–160.
6. Rubinson, K. A. (1981) Concerning the form of biochemically active vanadium. *Proc. Roy. Soc. Lond.* **B212,** 65–84.
7. Sánchez, I., Hughes, R. T., Mayer, B. J., et al. (1994) Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-Jun. *Nature* **372,** 794–798.
8. Kyriakis, J. M. and Avruch, J. (1990) pp54 MAP-2 kinase: a novel serine/threonine protein kinase regulated by phosphorylation and stimulated by poly-L-lysine. *J. Biol. Chem*. **265,** 17,355–17,363.
9. Liu, H., Nishitoh, H., Ichijo, H., and Kyriakis, J. M. (2000) Activation of apoptosis signal-regulating kinase-1 (ASK1) by TNF receptor-associated factor-2 requires prior dissociation of the ASK1 inhibitor thioredoxin. *Mol. Cell. Biol*. **20,** 2198–2208.
10. Rana, A., Gallo, K., Godowski, P., et al. (1996) The mixed lineage protein kinase SPRK phosphorylates and activates the stress-activated protein kinase activator. SEK1. *J. Biol. Chem.* **271,** 19,025–19,028.
11. Yan, M., Dai, T., Deak, J. C., et al. (1994) Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1. *Nature* **372,** 798–800.
12. Dérijard, B., Hibi, M., Wu, I.-H., et al. (1994) JNK1: A protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun transactivation domain. *Cell* **76,** 1025–1037.
13. Pandey, P., Raingeaud, J., Kaneki, M., et al. (1996) Activation of p38 mitogen-activated protein kinase by c-Abl-dependent and -independent mechanisms. *J. Biol. Chem.* **271**, 23,755–23,779.
14. Makkinje, A., Quinn, D. A., Chen, A., et al. (2000) Gene 33/Mig-6, a transcriptionally inducible adapter protein that binds GTP-Cdc42 and activates SAPK/JNK. *J. Biol. Chem.* **275,** 17,838–17,847.

15. Shapiro, P. S., Evans, J. N., Davis, R. J., and Posada, J. A. (1996) The seven transmembrane-spanning receptors for endothelin and thrombin cause proliferation of airway smooth muscle cells and activation of the extracellular signal regulated kinase and c-Jun NH_2-terminal kinase groups of mitogen-activated protein kinases. *J. Biol. Chem.* **271,** 5750–5754.
16. Pombo, C. M., Bonventre, J. V., Avruch, J., Woodgett, J. R., Kyriakis, J. M., and Force, T. (1994) The stress-activated protein kinases are major c-Jun amino-terminal kinases activated by ischemia and reperfusion. *J. Biol. Chem.* **269,** 26,546–26,551.

5

Investigating the Cellular BMK1/ERK5 Signaling Pathway

Richard I. Tapping, Kato Yutaka, Ta-Hsiang Chao, Masaaki Hayashi, Jeng-Fan Lo, Sung-Woo Kim, and Jiing-Dwan Lee

1. Introduction

Big mitogen-activated protein kinase 1 (BMK1), also known as extracellular signal-regulated kinase 5 (ERK5), is the most recently identified member of the mammalian mitogen-activated protein kinase (MAPK) family *(1,2)*. Cellular activation of BMK1 is induced by a variety of stimuli including growth factors, oxidative stress, and hyperosmolar conditions *(3–7)*. These physiologic mediators activate cellular BMK1 through the direct and sequential activation of a signaling cascade comprising the kinases MEKK3, MEK5, and BMK1 *(4,5,8–10)*. We have shown that the activation of this signal transduction pathway is required for growth factor-mediated cell proliferation and cell-cycle progression *(5,11)*.

Similar to other MAPKs, BMK1 functions through the phosphorylation and activation of specific downstream targets. We have identified certain members of the MEF2 family of transcription factors, which regulate growth factor-induced early gene expression, as direct downstream targets of BMK1 activity *(4,12)*. In addition, we have identified serum and glucocorticoid-inducible kinase as another target directly phosphorylated by activated BMK1. Furthermore, we have demonstrated that the phosphorylation of these targets by BMK1 is required for BMK1-mediated cell-cycle progression *(4,11)*.

Our studies of BMK1 have revealed physiologic agonists for its activation, intracellular regulators of its activity, as well as downstream targets that are essential for its function in controlling cell proliferation and cell-cycle progression. A number of specific molecular reagents and experimental methods have been indispensable for our studies of BMK1. These include constitutively active kinases, dominant-negative kinases, as well as direct and indirect assays for the measurement of BMK1 activity in cells. Here, we provide a practical
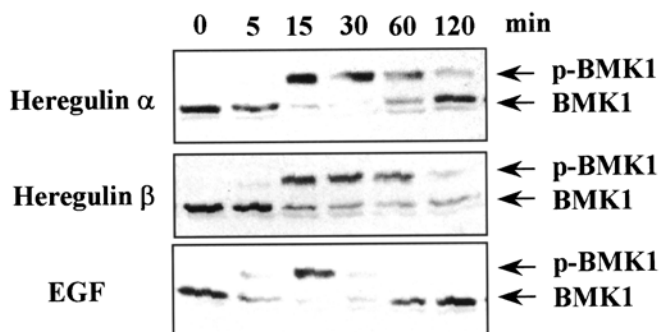
Fig. 1. Detection of endogenous activated BMK1. T47D cells incubated in serum-free medium were treated with either 1 n*M* heregulin α, 1 n*M* heregulin β, or 1 ng/mL of EGF for various times, as indicated. Endogenous activated BMK1 was detected from cell lysates by Western blot as described in the text. p-BMK1 denotes the phosphorylated form of BMK1 whose activity peaks at 15 min poststimulation.

summary of these reagents and methods, which we hope will provide tools for attaining a broader understanding of the mechanism of action and cellular functions of this important MAPK.

1.1. Detection of Endogenous BMK1 Activation

Novel physiologic agonists or upstream regulators of BMK1 have been revealed through the use of a sensitive Western blot assay that detects the activated form of endogenous BMK1. This assay is based on the observation that activated BMK1 exhibits a measurably slower migration than that of the inactive kinase in a 6% SDS-PAGE gel (*see* **Note 1**). This assay has been successfully utilized in defining growth factors such as epidermal growth factor (EGF), heregulins, and nerve growth factor (NGF) as physiologic stimulators of BMK1. In addition, this assay has successfully identified MEKK3 and MEK5 as upstream regulators of the BMK1 signaling pathway (*see* **Fig. 1**).

1.2. Selective Cellular Activation or Inhibition of BMK1

To investigate the biological function and the cellular targets of BMK1, we have devised a method to selectively activate or inhibit endogenous BMK1 activity by creating dominant-active or dominant-negative forms of the upstream MAPK kinase (MAPKK) MEK5. It is established that the activation of a MAPKK is through dual phosphorylation within kinase subdomain VIII. Therefore, we have introduced negatively charged aspartic acid residues in place of the natural phosphorylation sites within kinase subdomain VIII of MEK5 to make a dominant-active form of this kinase called MEK5(D) (*see*
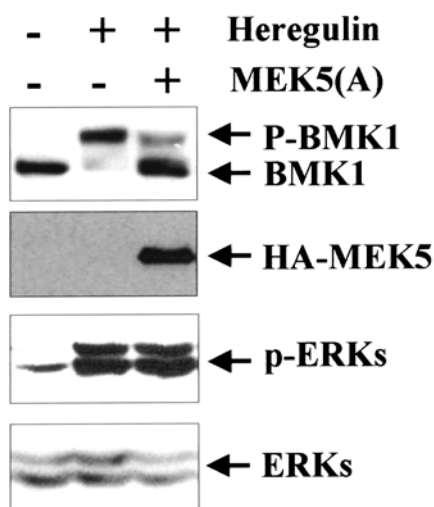
Fig. 2. Dominant-negative MEK5(A) is a specific inhibitor of BMK1 activation. MCF7 cells were transfected with either empty vector or MEK5(A) as indicated followed by stimulation with 1 n*M* heregulin in serum-free medium for 15 min. MEK5(A) greatly diminishes the level of heregulin-induced BMK1 phosphorylation (P-BMK1) without any measurable effect on the level of heregulin-induced ERK phosphorylation.

Note 2). Conversely, we have introduced alanines into the same sites to make a dominant-negative form called MEK5(A) (*see* **Note 3**). MEK5(A) cannot be phosphorylated or activated by its upstream kinase MEKK3. Both MEK5(A) and MEK5(D) have been used to identify new cellular substrates and to assess cellular actions mediated by BMK1 in response to different agonists *(4,5)* (*see* **Fig. 2**).

1.3. BMK1 Reporter Gene Assay

The activity of BMK1 derived from cell extracts has been measured in vitro by a standard immune complex kinase assay using either myelin basic protein (MBP) or MEF2C protein as substrates *(4)*. However, the in vivo activity of BMK1 can be directly assessed using a simple reporter gene assay. In this assay, the transactivation domain of SAP1a, an established target of BMK1, has been fused in frame with the DNA-binding domain (DBD) of the yeast transcription factor GAL4. The activity of this GAL4 fusion protein is then measured in cells by cotransfecting a reporter construct comprising five copies of the GAL4-binding site upstream of a luciferase gene *(4s12)*. A truncated form of SAP1a missing the C-terminus—amino acid residues 1–267 of SAP1a fused in frame with the GAL4 DBD—is used as a negative control in this assay (*see* **Fig. 3**).
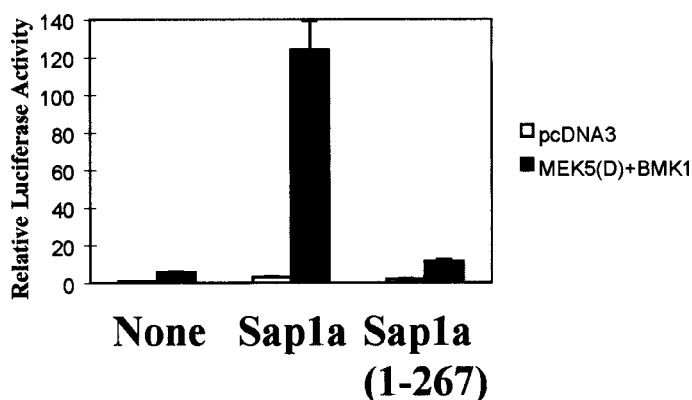
Fig. 3. Sap1a is a reporter of BMK1 activation. HeLa cells were transfected with reporter plasmid PG5ElbLUC along with expression vectors encoding Sap1a or truncated Sap1a (1-267) as GAL4 fusion proteins. Some cells were also cotransfected with expression vectors encoding BMK1 and dominant-active MEK5(D) as indicated. Relative luciferase activities were measured 48 h after transfection as described in the text. All activities were normalized to cells transfected with luciferase reporter alone whose activity was taken as 1.

2. Materials

2.1. Western Blot Assay

1. Anti-BMK1 antibody: The kinase domains of MAPKs are highly conserved. However, BMK1 possesses a large nonkinase region at the C-terminus that has no similarity with any known proteins including other MAPKs. Therefore, we have generated a rabbit polyclonal anti-BMK1 antibody against the unique C-terminal region of BMK1. This polyclonal antibody was generated by injecting rabbits with amino acids 783–806 of BMK1 as a keyhole limpet hemocyanin fusion protein *(1,3)*.
2. 1X RIPA buffer: 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 1 m*M* phenylmethylsulfonyl fluoride in 1X phosphate-buffered saline (PBS).
3. 2X SDS loading buffer: 0.25 *M* Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 10% β-mercaptoethanol.
4. 1X Tris-buffered saline (TBS): 10 m*M* Tris-HCl, pH 8.0, 0.9% NaCl.
5. Blocking solution: 2% BSA and 0.3% Tween-20 in 1X TBS.
6. Washing solution: 1% BSA and 0.3% Tween-20 in 1X TBS.
7. Horseradish peroxidase (HRP)–conjugated protein G, 1 mg/mL (Pierce, Rockford, IL).

2.2. Mammalian Expression Vectors Encoding MEK5(D) or MEK5(A)

The dominant-active kinase MEK5(D) was generated by mutating both Ser311 and Thr315, comprising the dual phosphorylation site, to aspartic acid

through a polymerase chain reaction-based approach. Similarly, these two residues were mutated to alanine to generate the dominant negative kinase MEK5(A).

2.3. Cell Lines and Media

1. Cells: T47D and MCF7 mammary carcinoma cells as well as HeLa human cervical carcinoma cells were obtained from American Type Culture Collection, Rockville, MD.
2. Medium: Dulbecco's modfied Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS), 1% pen/strep.

2.4. Reporter Assays

1. Trans-reporter: The constructs encoding the GAL4-binding domain fused to Gal4/Sap-1a or Gal4/Sap1a (1–267) are as described previously *(13)* and were obtained from Dr. Janknecht (Salk Research Institute).
2. PG5ElbLUC: the GAL4-responsive reporter plasmid contains five GAL4 sites upstream of a minimal promoter driving the firefly luciferase gene *(14)*.

2.5. Recombinant Adenoviruses

To efficiently deliver MEK5(D) or MEK5(A) into mammalian cells, we generated recombinant adenoviruses encoding MEK5(D) or MEK5(A). These cDNAs were cloned into the *Hin*dIII/*Xba*I site of pAd/RSV and recombinant viruses were generated as described previously *(5)*. Recombinant adenoviral stocks of at least 10^{11} plaque-forming unit (PFU)/mL are produced in 293 cells and purified by density gradient ultracentrifugation *(5,15)*.

3. Methods

3.1. Detection of Endogenous BMK1 Activation

1. Seed T47D cells in six-well plates overnight to achieve 40% confluency. The next day, starve the cells in growth factor-deficient medium (DMEM without FCS) for 16 h. Separately add 1 n*M* heregulin α1, 1 n*M* heregulin β1, or 1 ng/mL of EGF to each well of starved cells for the time intervals 0, 5, 15, 30, 60, and 120 min.
2. Place the treated cells on ice, wash once with ice-cold PBS, and subsequently lyse with 200 μL of RIPA buffer for 10 min on ice. Microfuge the cell lysates for 10 min at 14,000 rpm, to remove unlysed cell debris, and transfer the supernants to fresh eppendorf tubes. Determine the protein concentration of each cell lysate by standard bicinchoninic acid (BCA) assay (Pierce).
3. Mix a volume of cell lysate, representing 50 μg of total protein, with an equal volume of 2X SDS loading buffer and boil at 100°C for 10 min. Load the samples on a 6% SDS-polyacrylamide gel electrophoresis (PAGE) gel and run until the bromophenol blue dye reaches the bottom of the gel.
4. Electroblot the gel onto nitrocellulose paper and block with blocking buffer for 1 h at room temperature. Incubate the blot with blocking buffer containing a 1:5000 dilution of anti-BMK1 antibody for 3 h at room temperature with mild constant

agitation (*see* **Notes 4** and **5**). Wash the blot three times, for 15 min each, in washing buffer. Incubate the blot in blocking buffer containing a 1:5000 dilution of HRP-conjugated protein G for 30 min. Wash the blot three times, for 15 min each, in washing buffer.
5. Develop the blot using a standard enhanced chemiluminescence-based system.

3.2. Selective Inhibition of Cellular BMK1

1. Plate MCF7 cells overnight in six-well plates to achieve 40% confluency. The next day, treat the cells with or without recombinant adenovirus encoding MEK5(A) at a multiplicity of infection (MOI) of 10^3 PFU/cell for 16 h (*see* **Note 6**). Remove the recombinant viruses by replacing the medium with fresh medium and incubate for an additional 8 h. Starve the cells in a growth factor-deficient medium (DMEM without FCS) for 16 h and then treat with 1 n*M* heregulin β1 for 15 min.
2. Prepare cell lysates and perform electrophoresis and Western blot procedures to determine the activation of endogenous BMK1 as described in **Subheading 3.1.** Run a separate portion of each cell lysate on a 10% SDS-PAGE gel and electroblot. Determine the expression of HA-MEK5 using the anti-HA antibody HA.11 (Convance). Determine the expression of ERK1/2 using the anti-ERK antibody (Cell Signaling Technology). In addition, determine the activation of endogenous ERK1/2 using an antiphospho ERK antibody (Cell Signaling Technology).

3.3. Selective Cellular Activation of BMK1

1. Seed HeLa cells on six-well plates overnight to achieve 30% confluency. Transfect each well of cells with 1 μg of total plasmid DNA (e.g., 0.25 μg of MEK5(D), 0.25 μg of BMK1, 0.25 μg of Gal4/Sap-1a *[13]*, and 0.25 μg of PG5ElbLUC) using lipofectamine (Gibco-BRL) (*see* **Note 7**). After 6 h, replace the medium containing lipofectamine with fresh medium and incubate the cells for an additional 48 h.
2. Wash the cells once on ice with PBS and lyse using 200 μL of lysis buffer (Promega, Madison, WI) per well for 10 min. Spin the resulting cell lysate in a microfuge for 10 min at 14,000 rpm. Transfer the supernatant to a fresh tube, and assay a small aliquot of each lysate for luciferase activity using luciferin substrate (Promega) and a luminometer.
3. Determine the protein concentration of each lysate by standard BCA assay (Pierce), and use these values to normalize the luciferase activities (*see* **Note 8**).

4. Notes

1. We have confirmed that the shift of BMK1 mobility in a 6% SDS-PAGE gel is directly proportional to the degree of activation by comparing this shift with the activity of BMK1 in an immune complex kinase assay using MBP or MEF2C as a substrate *(4,5)*.
2. We have also generated a dominant-active form of MEK5 by replacing the dual phosphorylation sites of MEK5 with glutamic acids, MEK5(E) instead of aspar-

tic acid, MEK5(D). However, the ability of MEK5(E) to activate BMK1 is only about one third of that of MEK5(D) as determined by Western blot and in vitro kinase assay for BMK1.

3. BMK1(AEF), whose actual TEY dual phosphorylation site is replaced by AEF, is a dominant-negative form of BMK1 whose expression can also selectively block cellular BMK1 activity *(4,5)*.

4. We have found that anti-BMK1 antibody dilutions of less than 1:5000 increase the detection of endogenous BMK1; however, these lower dilutions also increase the presence of nonspecific background bands in this Western blot assay.

5. In addition to Western blotting, this BMK1 antibody is also useful for the immuno-precipitation and immunostainning of BMK1 in mammalian cells as described *(4,5)*.

6. To ensure that the observed results are not owing to infection by recombinant adenovirus, a control virus (generated by using empty vector) can be used as a negative control in this experiment.

7. In addition to SAP1a, we have shown that a GAL4 fusion of MEF2C is a useful reporter of BMK1 activity in this assay *(4)*.

8. β-Galactosidase activity from a cotransfected expression vector such as pCMVβ-gal can also be used, instead of total protein, to normalize cellular luciferase activities *(4,12)*.

Acknowledgments

References

1. Lee, J.-D., Ulevitch, R. J., and Han, J. (1995) Primary structure of BMK1: a new mammalian MAP kinase. *Biochem. Biophys. Res. Comm.* **213,** 715–724.

2. Zhou, G., Bao, Z. Q., and Dixon, J. E. (1995) Components of a new human protein kinase signal transduction pathway. *J. Biol. Chem.* **270,** 12,665–12,669.

3. Abe, J.-I., Kushuhara, M., Ulevitch, R. J., Berk, B. C., and Lee, J.-D. (1996) Big mitogen-activated protein kinase 1 (BMK1) is a redox-sensitive kinase. *J. Biol. Chem.* **271,** 16,586–16,590.

4. Kato, Y., Kravchenko, V. V., Tapping, R. I., Han, J., Ulevitch, R. J., and Lee, J.-D. (1997) BMK1/ERK5 regulates serum-induced early gene expression through transcription factor MEF2C. *EMBO J.* **16,** 7054–7066.

5. Kato, Y., .Tapping, R. I., Huang, S., Watson, M. H., Ulevitch, R. J., and Lee, J.-D. (1998) Bmk1/Erk5 is required for cell proliferation induced by epidermal growth factor. *Nature* **395,** 713–716.

6. Kamakura, S., Moriguchi, T., and Nishida, E. (1999) Activation of the protein kinase ERK5/BMK1 by receptor tyrosine kinases. Identification and characterization of a signaling pathway to the nucleus. *J. Biol. Chem.* **274,** 26,563–26,571.

7. Fukuhara, S., Marinissen, M. J., Chiariello, M., and Gutkind, J. S. (2000) Signaling from G protein-coupled receptors to ERK5/Big MAPK 1 involves Galpha q and Galpha 12/13 families of heterotrimeric G proteins: evidence for the existence of a novel Ras AND Rho-independent pathway. *J. Biol. Chem.* **275,** 21,730–21,736.

8. Chao, T. H., Hayashi, M., Tapping, R. I., Kato, Y., and Lee, J.-D. (1999) MEKK3 directly regulates MEK5 activity as part of the big mitogen-activated protein kinase 1 (BMK1) signaling pathway. *J. Biol. Chem.* **274,** 36,035–36,038.

9. English, J. M., Vanderbilt, C. A., Xu, S., Marcus, S., and Cobb, M. H. (1996) Isolation of MEK5 and differential expression of alternatively spliced forms. *J. Biol. Chem.* **270,** 28,897–28,902.

10. Sun, W., Kesavan, K., Schaefer, B. C., et al. (2001) MEKK2 associates with the adapter protein Lad/RIBP and regulates the MEK5-BMK1/ERK5 pathway. *J. Biol. Chem.* **276,** 5093–5100.

11. Hayashi, M., Tapping, R. I., Chao, T. H., et al. (2001) BMK1 mediates growth factor-induced cell proliferation through direct cellular activation of serum and glucocorticoid-inducible kinase. *J. Biol. Chem.* **276,** 8631–8634.

12. Kato, Y., Zhao, M., Morikawa, A., et al. (2000) Big mitogen-activated kinase regulates multiple members of the MEF2 protein family. *J. Biol. Chem.* **275,** 18,534–18,540.

13. Chang, M. W., Barr, E., Lu, M. M., Barton, K., and Leiden, J. M. (1995) Adenovirus-mediated over-expression of the cyclin/cyclin-dependent kinase inhibitor, p21 inhibits vascular smooth muscle cell proliferation and neointima formation in the rat carotid artery model of balloon angioplasty. *J. Clin. Invest.* **96,** 2260–2268.

14. Janknecht, R. and Hunter, T. (1997) Activation of the Sap-1a transcription factor by the c-Jun N-terminal kinase (JNK) mitogen-activated protein kinase. *J. Biol. Chem.* **272,** 4219–4224.

15. Gupta, S., Campbell, D., Derijard, B., and Davis, R. J. (1995) Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science* **267,** 389–393.

6

Pull-Down Assays for Guanoside 5'-Triphosphate-Bound Ras-Like Guanosine 5'-Triphosphatases

Miranda van Triest and Johannes L. Bos

1. Introduction

Small guanosine 5'-triphosphatases (GTPases) of the Ras superfamily switch between a guanosine 5'-diphosphate (GDP)-bound and a guanosine 5'-triphosphate (GTP)-bound conformation. This cycle is regulated by guanine nucleotide exchange factors that release the bound nucleotide. Since the concentration of GTP is about 10-fold higher than GDP, the empty binding site is rapidly filled with predominantly GTP. The conversion of GTP to GDP is facilitated by GTPase-activating proteins (GAPs), which contribute an extra catalytic group to the intrinsic GTPase activity of the proteins. In general, the GTP-bound form is the active form of the protein, and measurements of the ratio of GDP-GTP bound determines the activation state. Until recently the common method to measure the active GTP-bound form of small GTPases was immunoprecipitation of 32P- labeled protein followed by separation of the bound, radiolabeled GDP and GTP by thin-layer chromatography. This method allows determination of the percentage of GTP bound. This method still is the "gold standard," but it is technically demanding and needs relatively high levels of radioactivity. Furthermore, side effects owing to radiation-induced DNA damage cannot be excluded. For a large number of small GTPases, an additional and more serious problem is that suitable precipitating antisera are still unavailable. This implies that epitope-tagged GTPases have to be used to assess activation. This method may give false results owing to overexpression and the similarities among the various small GTPases.

Particularly for those GTPases lacking proper precipitating antisera, novel procedures needed to be developed *(1)*. These novel methods were based on the characteristic of small GTPases that only in the GTP-bound state would

2. Grow the transformed bacteria overnight at 37°C in 10 mL of LB medium in the presence of ampicillin (50 μg/mL) or kanamycin (25 μg/mL).

3. Dilute the bacterial culture 20 times in fresh LB medium and grow them for 1 h, after which the production of the probe is induced by adding 0.1 m*M* IPTG for 2–4 h.

4. Harvest the bacteria by centrifugation (10 min, 5000*g*), resuspend in 1/50 culture volume of ice-cold bacterial lysis buffer, and lyse by sonication.

5. Spin the lysate for 20 min at 40,000*g* to remove bacterial debris and collect the supernatant. Use the cleared lysate fresh or store it at –80°C after freezing in liquid nitrogen.

3.2. Cell Stimulation and Lysis

1. The day before the experiment, seed the cells such that they are 80–90% confluent at the day of the experiment. For each time point, use one 10-cm dish. Usually cells are serum-starved before the experiment to lower the basal level of activation, but this depends on the GTPase and the cell system used (*see* **Note 1**).

2. Stimulate the cells for the appropriate time. Stimulation is terminated by quick aspiration of the medium, after which the dishes are put on ice and washed with ice-cold PBS (*see* **Note 2**).

3. Add 600 μL of ice-cold cell lysis buffer to each 10-cm dish.

4. Scrape the cells from the dishes and collect the lysate in an Eppendorf tube.

5. Spin the lysates in a microcentrifuge for 5 min at 4°C to remove insoluble material. In the meantime, take new Eppendorf tubes and add 15 μL of a 1:1 slurry of glutathion-agarose beads in cell lysis buffer, together with approx 30 μg of GST-RBD probe (*see* **Notes 3** and **4**).

6. Transfer the cleared lysates to the new tubes with beads and probe. Save a small fraction (50 μL) of the lysate to determine total levels of the GTPase later.

7. Rotate the tubes for 60 min at 4°C to allow binding of the activated GTPase to the beads.

8. Wash the beads four times with cell lysis buffer. Remove the buffer carefully after the last washing step with a thin pipet tip, and add 20 μL of Laemmli sample buffer (*see* **Note 5**).

3.3. Western Blotting

1. Separate the precipitated proteins by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) together with the total lysate fraction. We routinely use minigels (Bio-Rad) of 13% polyacrylamide (*see* **Notes 6** and **7**).

2. Incubate a single fresh ampicillin-resistant colony in 50 mL of LB medium supplemented with 75 μg/mL of ampicillin overnight at 37°C in a shaker.
3. Dilute 20 mL of culture in 1 L of fresh LB medium containing 75 μg/mL of ampicillin and 0.4% D-glucose, and incubate at 37°C to OD_{600} of 0.6–0.7 (about 90 min).
4. Add IPTG to a final concentration of 0.1 mM to induce expression of the protein and continue incubation for an additional 2–3 h at 37°C.
5. Centrifuge the culture at 7700g and 4°C for 10 min to spin down the bacteria. At this stage the pellet can be stored overnight at –80°C. A small aliquot can be used to control for the induction of GST-fusion protein.
6. Resuspend the pellet in 25 mL of cold bacterial lysis buffer and sonicate 10 times for 30 s on ice (Utrasonic Processor UP200S, S7 tip, amplitude 40%).
7. Clear the lysate by centrifuging for 1 h at 12,000g and 4°C, and store the resulting cleared lysate in aliquots at –80°C; these will be stable for months. Multiple freeze-thawing should be avoided, however. GST-fusion protein present in the cleared lysate can be monitored by SDS-polyacrylamide gel electrophoresis.

The *E. coli* strain BL21 did not give very good yields of GST-RalBD. We therefore tested several bacterial strains and found that the protease-negative *E. coli* AD202 (*see* **ref. 7**) gave much better yields. The protocol is the same except that these transfected bacteria were grown in LB medium supplemented with 75 μg/mL of ampicillin, 25 μg/mL of kanamycin, 0.4% D-glucose, and 10% brain heart infusion medium (Difco, Detroit, MI). Incubation with IPTG is for 16 h at room temperature.

3.2. Identification of GTP-Bound GTPases With Activation-Specific Probes

1. Precipitate the GTP-bound GTPases from clear cell lysate using activation-specific probes that are precoupled to glutathion-agarose beads and detect by Western blotting using antibodies specific for a small GTPase.
2. Wash a 10% glutathion-agarose suspension two times with cell lysis buffer and add the cleared lysate containing the activation-specific probes and incubate 30–60 min on a tumbler at 4°C.
3. The beads with bound activation-specific probe were washed four times with cell-lysis buffer.
4. Usually for each sample to be analyzed, use 75 μL of a 10% suspension of glutathion-agarose beads precoupled to 100 μL of cleared lysate. This vast excess of activation-specific probe may be adjusted depending on the level of expression of the probe protein.
5. Put the culture dishes (usually 9-cm dishes) containing the cells for analysis that are usually incubated for a certain time with a stimulus on ice and wash two times with ice-cold PBS.
6. Add 1 mL of cold cell-lysis buffer and scrape the cells using a rubber policeman.
7. Transfer the crude lysate to an Eppendorf tube and centrifuge for 10 min at 14,000 rpm in an Eppendorf centrifuge at 4°C.
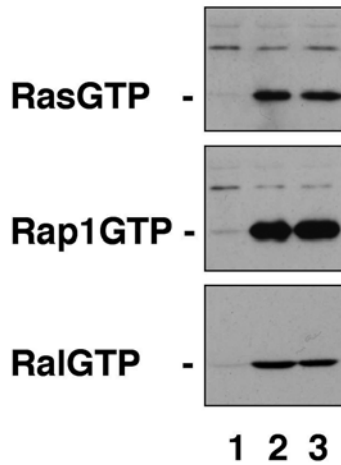
Fig. 1. Identification of activated Ras-like small GTPases. (**Top**) A14 cells were stimulated with insulin (5 μg/mL) for 0 (lane 1), 5 (lane 2), and 15 min (lane 3). Cells were lysed and RasGTP was collected with RafRBD. Ras was detected after Western blotting. (**Middle and bottom**) Human platelets were stimulated with α-thrombin (0.5 U/mL) for 0 (lane 1), 30 (lane 2), and 60 s (lane 3) and lysed. Rap1GTP (middle) and RalGTP (bottom) were detected by Western blotting.

8. Add the glutathion-agarose beads coupled with the activation-specific probe and incubate for 45 min on a tumbler at 4°C.
9. Wash the beads four times with cell-lysis buffer. After the final wash, carefully remove the remaining fluid with an insulin syringe, add 20 μL of Laemmli sample buffer and heat the sample for 5 min at 95°C.
10. Separate the sample on a 12.5% SDS-polyacrylamide gel and transfer to a PVDF membrane by electrophoresis.
11. Stain the membrane with Ponceau S to monitor for equal distribution of the GST-fusion protein over the different samples.
12. Incubate the membrane for 1 h at room at temperature in blocking buffer.
13. Incubate the membranes with PBS supplemented with blocking buffer (10% [v/v]) (not listed in **Subheading 2.**) Tween-20 (0.1% [v/v]), and one of the antibodies specific for the GTPase for the corresponding GTPase.
14. Incubate was overnight at 4°C.
15. Wash the membrane three times for 10 min at 4°C with PBS supplemented with Tween-20 (0.1% [v/v]), and incubated with secondary antibody in the same buffer for 1 h at 4°C.
16. After a further three, 5-min washes at 4°C, visualize bound secondary antibody by enhanced chemiluminescence (NEN) according to the manufacturer's protocol. **Figure 1** shows a typical example of the activation-specific probe assay for Ras, Rap1, and Ral.

4. Notes

1. The described assay is very rapid and easy and allows handling of many samples in a short period of time.
2. It has great advantages over the previous assay using radiolabeled extracts. In particular, it is nonradioactive and it does not require highly specific immunoprecipitating antibodies.
3. The method only determines the relative increase in the GTP-bound conformation of the GTPase. This increase can be determined rather accurately by a serial dilution strategy, but the total percentage of the GTPase in the GTP-bound form can only be guessed.
4. A more serious problem could be that if a GTPase is strongly bound to an effector, the GST probe may not bind and, thus, the measured increase may be an underestimate. However, in all cases in which we have compared the activation-specific probe assay with the assay using radiolabeled cells, we observed a good correlation, indicating that the fraction of an active GTPase is a stable complex that may be relatively low, or that there is sufficient probe available to compete for that activation.
5. A final possible problem could arise from GAPs that hydrolyze the GTP after lysis. However, we observed only GAP effects when the time between lysis of the cells, addition of the probe, and incubation was too long and when the reactions were not performed in the cold.

Acknowledgments

References

1. Franke, B, Akkerman, J. W. N., and Bos, J. L. (1997) Rapid Ca2+-mediated activation of Rap1 in human platelets. *EMBO J.* **16,** 252–259.
2. Luo, Z., Diaz, B., Marshall, M. S., and Avruch, J. (1997) An intact Raf zinc finger is required for optimal binding to processed Ras and for ras-dependent Raf activation in situ. *Mol. Cell. Biol.* **17,** 46–53.
3. Taylor, S. J. and Shalloway, D. (1996) Cell cycle-dependent activation of Ras. *Curr. Biol.* **6,** 1621–1627.
4. de Rooij, J. and Bos, J. L. (1997) Minimal Ras-binding domain of Raf1 can be used as an activation-specific probe for Ras. *Oncogene* **14,** 623–625.
5. Wolthuis, R. M. F., Franke, B., van Triest, M., et al. (1998) Activation of the small GTPase Ral in platelets. *Mol. Cell. Biol.* **18,** 2486–2491.
6. de Rooij, J., Boenink, N. M., van Triest, M., Cool, R. H., Wittinghofer, A., and Bos, J. L. (1999) PDZ-GEF1, a guanine nucleotide exchange factor specific for Rap1 and Rap2. *J. Biol. Chem.* **274,** 38,125–38,130.

7. Nakano, H., Yamazaki, T., Ikeda, M., Masai, H., Miyatake, S., and Saito, T. (1994) Purification of glutathione S-transferase fusion proteins as a non-degraded form by using a protease-negative E. coli strain, AD202. *Nucleic Acids Res*. **22,** 543,544.

7

Regulation of MAPK Cascades by Protein Tyrosine Phosphatases

Josema Torres, Carmen Blanco-Aparicio, and Rafael Pulido

1. Introduction

The degree of activation of mitogen-activated protein kinases (MAPKs) in response to extracellular stimuli is tightly regulated in vivo in a cell type- and stimulus-dependent manner, as the result of the coordinated function of protein kinases and phosphatases *(1)*. Activation of MAPKs requires phosphorylation by MAPK kinases on both the threonine and tyrosine MAPKs regulatory residues, located in the activation loop within the kinase domain VII *(2)*. On the other hand, dephosphorylation of any of these two residues is sufficient to inactivate the MAPKs. Thus, the protein serine/threonine phosphatases PP2A and PP2C can inactivate, in intact cells, the MAPKs extracellular signal-regulated kinase 1/2 (ERK1/2) and p38α, respectively *(3,4)*. Furthermore, a MAPK dual-specific phosphatase family has been characterized, whose members differentially dephosphorylate and inactivate distinct MAPKs *(5,6)*. Finally, the related protein tyrosine phosphatases (PTPs) PTP-SL and HePTP/LC-PTP inactivate the MAPKs by specific dephosphorylation of the phosphotyrosine regulatory residue *(7)*. In particular, PTP-SL dephosphorylates the MAPKs ERK1/2 and p38α (but not c-Jun N-terminal kinase [JNK]) after association through a 16 amino acid kinase interaction motif, located in the cytosolic regulatory domain of this PTP *(8–10)*. Here, protocols are described to assess the effects of PTPs on the tyrosine dephosphorylation of the MAPKs regulatory sites and on their kinase activities, both in intact cells and in vitro. Examples using the PTP-SL tyrosine phosphatase and the ERK2 and JNK1 MAPKs are provided. These protocols can be applied to assess the tyrosine phosphatase activity towards MAPKs of any other PTP.

2. Materials

All solutions are prepared in distilled, deionized MilliQ filtered water. Cell culture and transfection procedures require sterile conditions. ^{32}P-radioactive materials should be handled with caution, following the safety laboratory standards for protection and disposal of radioactive waste.

2.1. Analyzing Effects of PTPs on MAPK Activities in Intact Cells

1. Mammalian cell lines suitable for transfection and transient overexpression of recombinant proteins.
2. cDNAs of the tagged-MAPKs and PTPs of interest, cloned into mammalian expression vectors.
3. Anti-tag, anti-MAPKs and anti-PTP antibodies, and protein A-Sepharose.
4. Tissue culture media, transfection reagents, and cell-activating agents.
5. Lysis buffer: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40 (IGEPAL CA-630), 2 mM Na$_3$VO$_4$, 100 mM NaF, 20 mM Na$_4$P$_2$O$_7$, 1 mM PMSF, 1 μg/mL of aprotinin.
6. Washing buffer: 20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerin, 10 mM Na$_4$P$_2$O$_7$.
7. Kinase reaction buffer: 20 mM HEPES, pH 7.5, 10 mM MgCl$_2$, 1 mM dithiothreitol (DTT), 2 mM Na$_3$VO$_4$, 3 μM cold adenosine triphosphate (ATP).
8. [γ-^{32}P]ATP.
9. 2X Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer: 100 mM Tris-HCl, pH 6.8, 4% bromophenol blue, 20% glycerin.

2.2. Analyzing Phosphatase Activity of a PTP Toward Activated MAPKs In Vitro

1. Purified recombinant PTP, catalytically active.
2. cDNAs of the tagged-MAPKs of interest, cloned into mammalian expression vectors.
3. Antiphosphoactive MAPKs, antiphosphotyrosine, and anti-MAPKs antibodies.
4. Tissue culture media, transfection reagents, and cell-activating agents.
5. Lysis buffer: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40 (IGEPAL CA-630), 2 mM Na$_3$VO$_4$, 100 mM NaF, 20 mM Na$_4$P$_2$O$_7$, 1 mM PMSF, 1 μg/mL of aprotinin.
6. Washing buffer: 20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerin, 10 mM Na$_4$P$_2$O$_7$.
7. Phosphatase reaction buffer: 25 mM HEPES, pH 7.5, 5 mM EDTA, 10 mM DTT (add the DTT fresh).
8. Polyvinylidene fluoride (PVDF) protein transfer membranes.
9. Transfer buffer: 48 mM Tris base; 39 mM glycine, 0.037% SDS, 20% methanol.
10. Prestained molecular weight standard markers.
11. Peroxidase-conjugated secondary antibodies.
12. Incubation buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.05% Triton X-100, 0.25% gelatin.

13. Stripping solution: 65 mM Tris-HCl, pH 6.8, 2% SDS, 0.85% (v/v) 2-mercaptoethanol.
14. Western blotting chemiluminescence kit.

3. Methods

The purpose of these methods is to test whether a PTP is involved in the direct control of the activity of MAPKs. First, a protocol is provided in which plasmids encoding the tagged-MAPKs and the PTPs of interest are cotransfected into mammalian adherent cells, achieving transient overexpression of both molecules 24–72 h posttransfection; following cell stimulation and immunoprecipitation of the recombinant tagged-MAPKs, in vitro kinase assays are performed in the presence of specific substrates, which are resolved by SDS-PAGE and processed for quantification of the incorporated radioactivity (*see* **Subheading 3.1.**). Alternatively, the phosphorylation status of the immunoprecipitated tagged-MAPKs can be analyzed by immunoblot using specific antiphosphoactive MAPKs or antiphosphotyrosine antibodies (*see* **Subheading 3.2.**). Second, a protocol is described to assess in vitro the tyrosine phosphatase activity of a purified PTP toward activated MAPKs (*see* **Subheading 3.2.**).

3.1. Analyzing Effects of PTPs on MAPK Activities in Intact Cells

1. Plate the cells into six-well plates and perform transfections with (1) plasmid containing the tagged-MAPK, and (2) a mixture of plasmids containing the tagged-MAPK plus the PTP (*see* **Note 1**). To keep the total amount of DNA constant in all the wells, add the corresponding amount of empty vector to those transfected only with the tagged-MAPK (*see* an example in **Fig. 1**).
2. After 24–72 h of culture, activate the cells with the appropriate stimulus and during the appropriate time (*see* **Note 2** and an example in **Fig. 1**). Keep points untreated as controls of basal MAP kinase activity.
3. Rinse the cells at the selected time of activation with ice-cold phosphate-buffered saline (PBS). For helpful technical instructions, *see* **Notes 3–5**.
4. Lyse the cells in ice-cold lysis buffer, and transfer the lysate to a 1.5-mL Eppendorf tube.
5. Centrifuge at 14,000g for 5 min, 4ºC. Save the supernatant and transfer to clean tubes.
6. *Optional:* Preclear the supernatant with 20 µL of protein A-Sepharose for 30 min at 4ºC under rotation. Centrifuge at 1500g for 1 min. Save the supernatant.
7. Measure the protein content using a Bradford assay. Keep 50–75 µg to monitor the expression of the recombinant proteins by immunoblot using the appropriate antibodies.
8. Add to the rest of the lysate the anti-tag antibody specific for the tagging of the recombinant MAPK. Incubate for 1–2 h on ice (for convenience, the antibody can be left overnight with the lysate at this step).
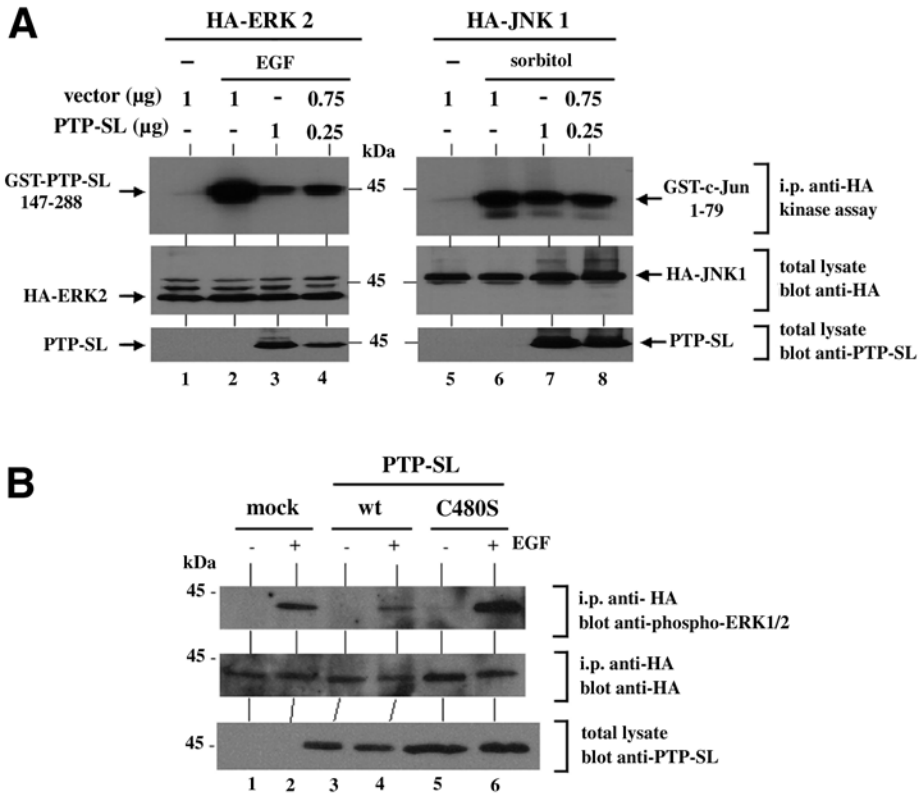
Fig. 1. **(A)** Effect of PTP-SL on activity of the MAPKs ERK2 and JNK1 in intact cells. HEK293 cells, plated on six-well plates, were transfected with plasmids encoding HA-tagged ERK2 (pCDNA3-HA-ERK2) or JNK1 (pRSα-HA-JNK1) (1 μg/well) plus the indicated amounts of pRK5-PTP-SL 147-549 (PTP-SL) or empty vector. After 48 h, cells were left untreated (lanes 1 and 5) or treated with 50 ng/mL of EGF for 5 min (for HA-ERK2, lanes 2–4) or with 0.5 *M* sorbitol for 30 min (for HA-JNK1, lanes 6–8). Cells were lysed, and the HA-tagged MAPKs were immunoprecipitated (400 μg of lysate/point) with the anti-HA 12CA5 monoclonal antibodies (MAbs). Immune pellets were subjected to an in vitro kinase assay using GST-PTP-SL 147-288 or GST-c-Jun 1-79 (1 μg/point) as the substrates for HA-ERK2 and HA-JNK1, respectively. The samples were resolved on 10% SDS-PAGE, followed by autoradiography (upper panels). As controls of expression, 100 μg of the total lysate samples was analyzed by immunoblot using the anti-HA 12CA5 MAb (middle panels) or an anti-PTP-SL antibody (lower panels). **(B)** Effect of PTP-SL on the phosphorylation of phosphoactive residues of ERK2. COS-7 cells, plated on six-well plates, were transfected with pCDNA3-HA-ERK2 (2 μg/well) plus empty vector (mock, lanes 1–2) or pRK5-PTP-SL 147-549 wild type (wt) (lanes 3–4) or C480S catalytically inactive mutation (lanes 5–6) (1 μg/well). After 48 h, cells were left untreated (–) or treated

9. Add 20 μL of protein A–Sepharose beads (1:1 in PBS) for 1 h at 4ºC under rotation.
10. Wash four times with 1 mL of ice-cold washing buffer.
11. Wash once with 1 mL of of ice-cold kinase reaction buffer (without cold ATP). Carefully aspirate the supernatant and leave the Eppendorf tube containing the pellet at room temperature for 1 to 2 min.
12. Add to the pellet 20 μL of room temperature kinase reaction buffer containing the appropriate substrate protein (1–3 μg) and the [γ-^{32}P]ATP (1–5 μCi) for 20 min at room temperature under constant shaking (*see* **Note 6**).
13. Add 30 μL of 2X SDS-PAGE loading buffer.
14. Boil the Eppendorf tube for 2 min, spin, and load the mix in an SDS-PAGE gel (the acrylamide content will depend on the molecular weight of the substrate). Include a lane with molecular weight standard markers.
15. Run the gel, stain with Coomasie blue, and dry on a piece of Whatman paper.
16. Use the gel to expose an autoradiography film or a phosphorImager screen.
17. For quantification of incorporated counts per minute, scise out the substrate protein band from the dry gel and put it in an Eppendorf tube containing 1 mL of scintillation liquid. Count on a scintillation counter. Alternatively, dry gels can be processed for quantification using a phosporImager.

An example of the effect of the tyrosine phosphatase PTP-SL on the activity of HA-ERK2 and HA-JNK1 is shown in **Fig. 1A**. As observed, a dose-dependent inhibitory effect on HA-ERK2 activity, but not on HA-JNK1 activity, is observed on coexpression with PTP-SL, indicating the specificity of this PTP toward ERK2. Note that different stimuli and substrates are used for each MAPK (HA-ERK2: epidermal growth factor [EGF] and GST-PTP-SL 147-288; HA-JNK1: sorbitol and GST-c-Jun 1-79). The controls of the expression of HA-ERK2, HA-JNK1, and PTP-SL are also shown. Regarding specificity, (*see* also **Notes 7** and **8**).

To monitor the phosphoactive content of the MAPKs on coexpression with the PTP, follow the previously described method from **steps 1–9**; then, process the samples for immunoblot analysis using antiphosphoactive MAPKs or antiphosphotyrosine antibodies, as indicated in **Subheading 3.2.** An example of such analysis is provided in **Fig. 1B**. As shown, the phosphoactive content of HA-ERK2 is diminished on coexpression with active PTP-SL (wild type), but not on coexpression with a catalytically inactive PTP mutation (C480S). See the dominant-negative effect of the PTP-SL C408S mutation (*see* **Note 2** and **Fig. 2**).

Fig. 1. *(continued)* with 50 ng/mL of EGF (+) for 5 min. Cell lysates (2 wells/point) were immunoprecipitated with the anti-HA 12CA5 MAb, resolved on 10% SDS-PAGE, and subjected to immunoblot with an antiphosphoactive-ERK1/2 antibody (upper panel). To monitor the amount of HA-ERK2 in all points, the same blot was stripped and reprobed with the anti-HA MAb (middle panel). As control of PTP-SL expression, 10 μg of total lysate samples was analyzed by immunoblot using the anti-PTP-SL antibody (lower panel).
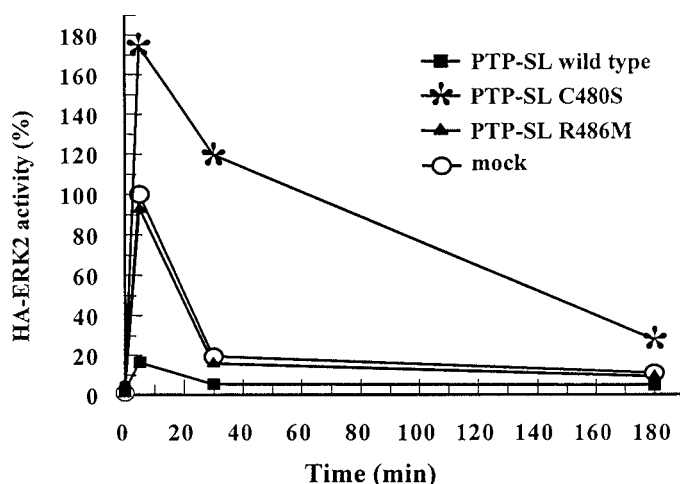
Fig. 2. Kinetics of activation by EGF of HA-ERK2 on coexpression with PTP-SL wild type or the catalytically inactive C480S or R486M mutants. HA-ERK2 and PTP-SL were coexpressed in COS-7 cells as in **Fig. 1**. After 48 h, cells were treated with 50 ng/mL of EGF during the indicated times, and the activity of HA-ERK2 was measured as described in **Subheading 3.1.**, using GST-PTP-SL 147-288 as the substrate. Data are presented as the percentage of HA-ERK2 activity with respect to that shown for cells transfected with pCDNA3-HA-ERK2 plus empty vector (mock) after 5 min of EGF stimulation (100% HA-ERK2 activity). Note the dominant-negative effect of the C480S trapping mutant, but not the R486M mutant.

3.2. Analyzing Phosphatase Activity of a PTP Toward Activated MAPKs In Vitro

1. Transfect cells with a plasmid containing the tagged-MAP kinase, treat the cells with the adequate stimulus, and immunoprecipitate the kinase with an anti-tag antibody. Alternatively, using a specific anti-MAPK antibody, the endogenous MAPK can be immunoprecipitated from nontransfected cells.
2. Perform the last washing of the immunoprecipitated pellets with 1 mL of ice-cold phosphatase reaction buffer.
3. Resuspend each immune complex pellet in 20 μL of room temperature phosphatase reaction buffer. Keep at room temperature.
4. Add increasing amounts of purified GST-PTP fusion protein. As a control, keep one point without GST-PTP. Incubate for 30 min at room temperature, under constant shaking (*see* **Notes 9** and **10**).
5. Add 30 μL of 2X SDS-PAGE loading buffer.
6. Boil the Eppendorf tube for 2 min, spin, and load the mix in an SDS-PAGE gel. Include a lane with prestained molecular weight markers.
7. Run the gel and transfer to a PVDF membrane. Cut a piece of the membrane in the range of 30–60 kDa.
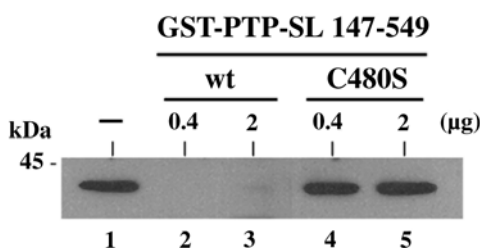
Fig. 3. Dephosphorylation of HA-ERK2 in vitro by PTP-SL. COS-7 cells, plated in six-well plates, were transfected with pCDNA3-HA-ERK2 (2 wells/point; 1 μg of DNA/well). After cell lysis and immunoprecipitation with an anti-HA MAb, immune complex pellets were resuspended in phosphatase reaction buffer in the presence of the indicated amounts of GST-PTP-SL 147-549 wt or C480S catalytically inactive mutant fusion proteins, as indicated, and subjected to phosphatase assays. In lane 1, no GST-fusion protein was added. Samples were resolved in 10% SDS-PAGE and processed for immunoblot with the antiphosphotyrosine 4G10 MAb.

8. Detect the phosphoactive MAPK by standard Western blot and chemiluminescence techniques, using an antiphosphoactive MAPK or an antiphosphotyrosine antibody as the primary antibody (*see* **Note 11**).

An example of the dephosphorylation of EGF-activated HA-ERK2 by purified GST-PTP-SL wild-type or catalytically inactive mutant is shown in **Fig. 3**.

4. Notes

1. There are different suitable protocols for transfection of adherent mammalian cells that work well with some given cell lines. If using vectors containing the SV40 origin of replication, COS-7 or HEK293 cells can be transfected with very high efficiency by the DEAE-dextran or the calcium phosphate methods, respectively. In addition distinct commercial lipids are available that can be used to transfect a wide range of cell lines. (For a detailed description of transfection procedures, *see* **ref. *11*.**) Be sure that, for the conditions of transfection, both the recombinant tagged-MAPK and the PTP are coexpressed in a representative number of cells. It is critical that most of the tagged-MAPK-positive cells are also positive for the PTP. Perform control transfections using distinct ratios of tagged-MAPK:PTP plasmid DNAs, and check coexpression of the two molecules by two-color immunofluorescence.
2. Activation of the distinct MAPKs is stimulus specific and, in most cases, time dependent. Hence, the selection of the appropriate time and stimulation conditions is important to test the effects of PTPs on the activity of the MAPKs. As an example, the activation kinetics of HA-ERK2 in response to EGF in COS-7 cells is shown in **Fig. 2**. Under this condition, HA-ERK2 follows a rapid and transient activation, reaching the peak of activation at about 5 min. When cotransfected

with PTP-SL, the activity of HA-ERK2 is decreased at all time points. As shown, the best time point to detect a clear inhibitory effect of PTP-SL is 5 min. When cotransfected with a catalytically inactive PTP-SL mutant (PTP-SL C480S), the inactivation of HA-ERK2 is delayed, as a consequence of a dominant-negative effect of this mutant (*see* also **Fig. 1B** and below); in this case, the best time point to observe the effect of PTP-SL on HA-ERK2 activity is 30 min.

Single amino acid substitutions of key residues involved in the catalysis of PTPs can be used that provide complementary information on the effects of the PTPs on their substrates *(12)*. Thus, additional evidence that a PTP is directly controlling the activity of a MAPK in intact cells is provided by the effects of catalytically defective PTP mutants on its activation. As an example, the effects on HA-ERK2 activation of PTP-SL catalytically inactive mutants are shown in **Fig. 2**. As shown, the PTP-SL C480S mutant, which is able to trap the physiologic PTP-SL substrate, acts as a dominant-negative mutant for HA-ERK2 activation; on the other hand, the catalytically inactive PTP-SL R486M mutant, which does not have the properties of a substrate-trapping mutant *(12)*, acts as a null mutant for HA-ERK2 activation. A dominant-negative effect on MAPK activation with the appropriate mutants is indicative of direct dephosphorylation of the MAPK by the PTP.

3. To prevent protein degradation and/or inactivation of the MAPKs, it is important to include in the lysis buffer a cocktail of protease and phosphatase inhibitors (freshly added), as indicated in **Subheading 2.1.**
4. Preclearing may help to reduce undesired background of nonspecific kinase activity.
5. To pipet small volumes of protein A-Sepharose beads, it is convenient to cut out the end of the micropipet tip.
6. The concentrations of radiolabeled and cold ATP in the reaction buffer may be changed to fit the desired conditions of the reaction and the specific activity of the MAPK under study.
7. To assess further the direct regulatory role of a PTP on MAPKs activation, the use of constitutively active mutant forms of MAPK kinases (the upstream activators of MAPKs), as the stimulus for MAPKs activation, is recommended. These experiments involve the transient coexpression of three molecules: the tagged-MAPK, the constitutively active MAPK kinase, and the PTP. If the PTP can inhibit the activation of the MAPK by its MAPK kinase constitutively active upstream activator, a direct effect of the PTP on the MAPK is likely to exist.
8. Since MAPKs form complexes with their activating and inactivating effectors by binding at specific docking sites *(13)*, an additional demonstration of the specificity of the PTP toward the MAPK can be obtained by analyzing the physical association between the two enzymes.
9. To obtain an optimal enzymatic activity, it is recommended that freshly purified preparations of GST-PTP fusion proteins dialyzed in phosphatase reaction buffer containing a reducing agent (10 m*M* DTT or 10 m*M* β-mercaptoethanol) be used. For a standard protocol of GST-fusion protein purification from *Escherichia coli*, see Subheading 3.1. in Chapter 3. Alternatively, other sources of purified PTP (recombinant or endogenous) are also suitable for this protocol.

10. As controls of specificity, the use of catalytically inactive PTP mutants is recommended. Additionally, the dephosphorylation of a specific substrate should be achieved at arelatively low dose (nanograms) of the purified PTP.

11. The heavy chains of the antibodies used in the immunoprecipitation of the MAPKs run about 50–55 kDa and could interfere with the detection of the MAPK phosphocontent, in cases in which the MAPK runs in the same region. This could be solved using the appropriate nonreactive secondary antibody.

Acknowledgments

References

1. English, J., Pearson, G., Wilsbacher, J., Swantek, J., Karandikar, M., Xu, S., and Cobb, M. H. (1999) New insights into the control of MAP kinase pathways. *Exp. Cell Res.* **253,** 255–270.

2. Marshall, C. J. (1995) MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. *Curr. Opin. Genet. Dev.* **4,** 82–89.

3. Alessi, D. R., Gomez, N., Moorhead, G., Lewis, T., Keyse, S. M., and Cohen, P. (1995) Inactivation of p42 MAP kinase by protein phosphatase 2A and a protein tyrosine phosphatase, but not CL100, in various cell lines. *Curr. Biol.* **5,** 283–295.

4. Takekawa, M., Maeda, T., and Saito, H. (1998) Protein phosphatase 2Cα inhibits the human stress-responsive p38 and JNK MAPK pathways. *EMBO J.* **17,** 4744–4752.

5. Keyse, S. M. (1998) Protein phosphatases and the regulation of MAP kinase activity. *Semin. Cell Dev. Biol.* **9,** 143–152.

6. Camps, M., Nichols, A., and Arkinstall, S. (2000) Dual specificity phosphatases: a gene family for control of MAP kinase function. *FASEB J.* **14,** 6–16.

7. Saxena, M. and Mustelin, T. (2000) Extracellular signals and scores of phosphatases: all roads lead to MAP kinase. *Semin. Immunol.* **12,** 387–396.

8. Pulido, R., Zúñiga, Á., and Ullrich, A. (1998) PTP-SL and STEP protein tyrosine phosphatases regulate the activation of the extracellular signal-regulated kinases ERK1 and ERK2 by association through a kinase interaction motif. *EMBO J.* **17,** 7337–7350.

9. Zúñiga, Á., Torres, J., Úbeda, J., and Pulido, R. (1999) Interaction of MAP kinases with the kinase interacion motif of the tyrosine phosphatase PTP-SL provides substrate specificity and retains ERK2 in the cytoplasm. *J. Biol. Chem.* **274,** 21,900–21,907.

10. Blanco-Aparicio, C., Torres, J., and Pulido, R. (1999) A novel regulatory mechanism of MAP kinases activation and nuclear translocation mediated by PKA and the tyrosine phosphatase PTP-SL. *J. Cell Biol.* **147,** 1129–1135.

11. Kingston, R. E., Chen, C. A., Okayama, H., and Rose, J. K. (1997) Transfection of DNA into eukaryotic cells, in *Current Protocols in Molecular Biology.*

(Ausubel, F. M., Brent, R., Kingston, R. E., et al., eds.), John Wiley & Sons, New York, pp. 9.0.1–9.17.3.

12. Flint, A. J., Tiganis, T., Bardford, D., and Tonks, N. K. (1997) Development of "substrate-trapping" mutants to identify physiological substrates of protein tyrosine phosphatases. *Proc. Natl. Acad. Sci. USA* **94,** 1680–1685.

13. Tanoue, T., Adachi, M., Moriguchi, T., and Nishida, E. (2000) A conserved docking motif in MAP kinases common to substrates, activators and regulators. *Nat. Cell Biol.* **2,** 110–116.

8

Use of Inhibitors in the Study of MAPK Signaling

Yoav D. Shaul and Rony Seger

1. Introduction

   To survive and execute their functions, cells need to respond to many extracellular signals such as soluble molecules, neighboring cells, and physical changes in the environment. When interacting with the cells, these extracellular signals cause many intracellular changes, which are executed through target molecules and gene expression. Therefore, the extracellular signal must be transferred from the extracellular moiety to the nucleus through several communication lines, which generally transmit the signal through a series of protein phosphorylation processes (for more details see Chapter 1 and references therein).

1.1. MAPK Signaling Cascades

   One of the major signaling families that participates in the intracellular transmission of extracellular signals is the group of mitogen-activated protein kinases (MAPKs), which is composed of serine/threonine kinases that phosphorylate and activate each other (reviewed in **refs. *1*** and ***2***). Todate, four distinct MAPK cascades that all contain a Thr-Xaa-Tyr motif have been identified and named according to the components in the MAPK level *(1,3)*. These cascades are: extracellular signal-regulated kinase (ERK), Jun N-terminal kinase (JNK; SAPK1), p38MAPK (p38; SAPK2-4), and Big MAPK (BMK, also known as ERK5). Additional MAPKs termed ERK7 and ERK8 also contain the Thr-Xaa-Tyr motif; however their mechanism of activation is not yet elucidated *(4,5)*. Even though the MAPK signaling cascades contain similar regulatory motifs, they differ in their physiologic activities. The ERK1/2 group usually plays a role in proliferation and differentiation, and so does the BMK. BMK is also involved in the response to stress, which seems to be the main

function of the p38MAPK and JNK groups. Regulation of the different cascades and the differences among them are described in other chapters.

1.2. Direct and Indirect Inhibition of MAPK

One of the main functions of signal transduction research is to understand the mode of regulation of various cellular processes by signaling modules. In other words, it is important to understand the downstream effects of different signaling pathways and the cellular responses that they regulate. The most common way to determine the involvement of a pathway in the studied cellular process is by blocking its activity followed by determining the effects of this blockade on the cellular readout. Besides the importance for research, the role of MAPKs in many severe diseases makes them excellent targets for pharmacologic modulation. Thus, the ERK1/2 cascade has been demonstrated to play a role in cellular transformation, diabetes mellitus, as well as in the repair of gastric ulcers and acute ischemic stroke *(6)*. To reduce the disease-related signaling through the ERK1/2 cascade, many studies have focused on the upstream activators of ERKs including Ras, Raf1, and MEK1/2 *(7–9)*. Many of the inhibitors identified have advanced to the stage of human clinical trials with only moderate success. A similar situation was reported for the other MAPK cascades, and, therefore, this type of "signal transduction therapy" still needs further development.

1.3. MAPK Inhibitors

Over the past years several methods to block intracellular signaling cascade have been developed. One way is the use of inactive forms of signaling components that act as dominant negative. This means that these mutants divert signals to their direction, which becomes a "dead end," thereby reducing the amount of signals that are diverted through the endogenic, wild-type protein. Other methods of inhibition are the use of small drugs as well as the use of nonrelated extracellular ligands to modulate intracellular signaling *(10)*. Although all three methods can be used in research and even in medicine, the use of small drugs is by far faster, cheaper, and convenient. Another advantage of such inhibitors is that they can be added to every cell and do not require exogenous protein expression.

1.4. MEK1/2 Inhibitors

The ERK cascade is the main MAPK unit to be activated upon growth factor stimulation. The components of this cascade are Ras; Raf-1; MEK1/2; ERKs; and several MAPK-activated protein kinases (MAPKAPKs) including ribosomal S6 kinase (RSK), MAPK signal-interacting kinase, and MAPK/SAPK-activated kinase. The MEKs are very specific to the native form of ERK1/2,

and this is can be one of the reasons for the relatively high number of inhibitors identified to the MEKs. During initial studies in the field, inhibition of the Ras-ERK pathway was achieved by dominant-negative forms or antisense constructs of the various components *(11,12)*. Using these techniques, it was shown that the full activity of the Ras-ERK pathway is essential for proliferation, differentiation, and oncogenic transformation in many cell types. Therefore, it was only a matter of time until the first inhibitory small molecule was identified. This molecule, [2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one], was termed PD98059 *[13]*) and shown to act directly on MEK1/2. Inhibition studies revealed that the compound is specific to MEK1/2 and in high concentration also to MEK5, but it does not inhibit the JNK and p38MAPK cascades *(14)*. The inhibition of MEKs may be problematic in studies of ERK1/2 in proliferation and oncogenic signaling. Another problem with this compound is its relative low solubility, which does not exceed 30 μg/mL in aqueous solution, and is not always sufficient to completely inhibit MEK1/2 in activated cells *(13)*. On the other hand, (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene), termed U0126 *(15)*, which is another MEK1/2 inhibitor that is often used in basic research, is soluble in much higher concentrations and is able to completely inhibit MEK activity under most conditions. This compound was identified through a high-throughput screen as a functional inhibitor of activator protein-1-driven gene activation *(15)*, and its characterization revealed that it operates through a reversible, noncompetitive inhibition of MEK1/2. Similar to PD98059, this inhibitor is selective to MEK1/2 and less to MEK5. In addition, these two inhibitors seem to have a similar mode of action since both of them inhibit the activation of MEKs and not the activity of MEKs toward ERKs, as was originally suggested *(16)*.

1.5. MEK Inhibitors in Clinical Trials

In addition to the mentioned compounds that are used primarily in in vitro studies, there are a few Raf-1 and MEK inhibitors that are now being tested as anticancer drugs in animals and human. For example, [2-(2-chloro-4-iodo-phenylamino)-*N*-cyclopropylmethoxy-3, 4-difluoro-benzamide], termed PD 184352 *(17)*, was shown to be a specific MEK1/2 inhibitor with good solubility and low IC$_{50}$ that is able to completely inhibit the activity of MEKs under most in vitro and cell culture conditions. Treatment of colon-26 tumor-implanted mice with PD184352 completely suppressed ERK phosphorylation and significantly inhibited tumor growth and invasiveness in these mice *(17)*. In addition, BAY 43-9006, which was identified as a Raf-1 inhibitor in a high-throughput screen, is another inhibitor of the ERK cascade *(18)*. BAY 43-9006 seems to have a mode of action similar to that of U0126 and was shown to significantly inhibit tumor growth in soft agar and in nude mice. To date, this

compound is being used in clinical trials, and investigators have reported a significant inhibition of stimulated ERK *(10)*. Taken together, all the inhibitors identified seem to be specific to MEKs, but not to ERK, Raf, or MAPKAPKs, indicating that the MAPKK level of the cascade is the most susceptible to inhibition and should be a good target for rational drug design.

1.6. p38MAPK and JNK Inhibitors

Another member of the MAPK family is the p38MAPK, which is a major signal transducer of stress stimuli and cytokines *(19–22)*. Identification of the first inhibitor for p38MAPK proceeded that of the kinase. [4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) 1H-imidazole], termed SB203580 *(19,21)* was first identified as an inhibitor of lipopolysaccharide-induced tumor necrosis factor α and interleukin-1β production in monocytes. Only later, using a column of the inhibitor, p38MAPK was identified and cloned. This p38MAPK inhibitor was demonstrated to specifically inhibit the activity of p38MAPK α- and β-isoforms *(19,21)* through a competitive mechanism *(23)*. However, p38MAPK γ- and δ-isoforms as well as all other protein kinases examined are not inhibited by the compound, and it was shown that this specificity is exerted from methionine 106, which seems to be unique to the sequence of modified SB203580 kinases *(24)*. Although specific in lower concentrations (10 μM), note that high concentrations of SB203580 (>20 μM) may interfere with activity of other kinases, including activation of Raf-1 *(25)*, and by an unidentified mechanism also of JNK and nuclear factor-κB *(26)*. Other inhibitors of p38MAPK that were recently developed are PD169316, SB203580, and SC68376 (*see* **Table 1**), all of which seem to be selective to p38MAPKα and β, although their specificity is still under examination. Another drug, VX-745 *(27)*, which is also selective to p38MAPKα and β, was introduced as a potential antiinflammatory drug and tested in a phase II trial. However, the results of the trial were disappointing mainly because of many side effects of the drug. Nonetheless, p38MAPK is considered to be a good target for several diseases such as inflammatory rheumatoid arthritis, and, therefore, many other inhibitors are likely to emerge in the future *(20)*.

JNK is stimulated upon stress, inflammatory cytokines, bacterial endotoxin, and ultraviolet radiation *(1,28)*. An inhibitor of JNK was developed only recently, through an in vitro screen in which any alteration in the phosphorylation of GST-c-Jun by JNK. This inhibitor is [anthra(1,9-cd)pyrazol-6(2H)-one], termed SP600125 *(28)*, which competes on the adenosine triphosphate binding site of JNK through a reversible interaction. This compound was shown to inhibit all three JNK isoforms with a similar potency, and to exhibit greater than 300-fold selectivity as compared to MAPKs *(28)*. Interestingly, another specific inhibitor of the JNK pathway was identified a few years ago by modi-

fying the structure of the neuroprotective TrkA inhibitor K252a. One of the derivatives studied was CEP1347, which retained its neuroprotective activity by selectively inhibiting MLK3 rather than TrkA *(29)*. Note, however, that CEP1347 is not a genuine JNK inhibitor because it inhibits the whole MAP3K family of MLK3 including MLK3, MLK2, MLK1, dual leucine zipper kinase, and leucine zipper kinase *(30)*. This family responds to a limited number of extracellular stimuli, which may also be involved in the activation of other cascades and the CEP1347 was shown to stimulate other signaling pathways *(31)*. Nevertheless, CEP1347 is now successfully examined for its neuroprotective effects in animals and humans. Other inhibitors that are helpful in the study of MAPK cascades are discussed in **Subheading 4.**

2. Materials

The solutions should be prepared in distilled/deionized water.

2.1. Cell Culture and Protein Extraction

1. Dulbecco modified Eagle's medium (DMEM) (#41965-039; Gibco-BRL).
2. Fetal calf serum (FCS) (#101-06078; Gibco-BRL), glutamine solution (Biological Industries, Beit Haemek, Israel), and antibiotics (Biolab, Jerusalem, Israel) stored in aliquots at –20°C.
3. Trypsin-EDTA (#T-3924; Sigma).
4. Stimulant: 50 µg/mL of epidermal growth factor (EGF) (#E-9644; Sigma) in EGF buffer (phosphate-buffered saline [PBS] containing 0.5 mg/mL of bovine serum albumin [BSA] [#A-9647; Sigma]).
5. 10X PBS, calcium and magnesium free (#14200-067; Gibco-BRL). Prepare 1X ice-cold PBS.
6. Homogenization buffer (buffer H) with protease inhibitors: 50 mM β-glycerophosphate (#G-6251; Sigma), pH 7.3, 1.5 mM EGTA, 1.0 mM EDTA, 1.0 mM dithiothreitol (DTT) (#D-9779; Sigma), 0.1 mM sodium orthovanadate, 1.0 mM benzamidine (#B-6506; Sigma), 10 µg/mL of aprotinin (#A-1153; Sigma), 10 µg/mL of leupeptin (#L-0649; Sigma), 2.0 µg/mL of pepstatin-A (#P-4265, Sigma).
7. Buffer A: 50 mM β-glycerophosphate, pH 7.3, 1.5 mM EGTA, 1.0 mM EDTA, 1.0 mM DTT, 0.1 mM sodium orthovanadate. Prepare a 10X stock solution (without DTT) and store at –20°C. Prior to use, add freshly prepared DTT.
8. Bradford reagent (Coomassie protein assay reagent, #BH44587; Pierce).

2.2. Inhibitors

See **Tables 1** and **2** and **Note 1** for inhibitors.

2.3. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

1. Gel electrophoresis apparatus and power supply.

Table 1
MAPK Inhibitors and Their Suppliers

Compound	Inhibitors	Company (cat. no.)	IC_{50}/working condition
Specific inhibitors			
PD98059	MEK1/2	Calbiochem (513000), BioMol (EI-360), Sigma (p-215), Promega (V1191)	2 μM/25 μM
U0126	MEK1/2	Calbiochem (662005), Sigma (U-120), BioMol (EI-282), Promega (V1121)	1 μM/5 μM
PD169316	P38 MAPK (α + β)	Calbiochem (513030)	89 nM/10 μM
SB202190	P38 MAPK (α + β)	Calbiochem (559388), BioMol (EI-294)	0.6 μM/10 μM
SB203580	P38 MAPK (α + β)	Calbiochem (559389), Sigma (S-8307), BioMol (EI-286), Promega (V1161)	70 nM/100 μM
SC68376	P38 MAPK (α + β)	Calbiochem (565625)	2–5 μM/20 μM
SP600125	JNK	BioMol (EI-305)	0.04 μM/5 μM
Less specific inhibitors			
CEP-1347 (KT7515)	MLK-3	Cephalon	23 nM/0.5 μM
Olomoucine	P34cdk1, p33cdk2, p33cdk5, ERK1/2	Calbiochem (495620), BioMol (CC-200), Promega (V2372/2373)	25 μM/300 μM
AG-126	Prevents ERK1/2 phosphorylation	Calbiochem (658452), Sigma (T-9177), BioMol (EI-263)	15.38 μM/50 μM

2. 4X Laemmli reducing sample buffer: 0.2 *M* Tris-HCl, pH 6.8, 40% (v/v) glycerol; 8% (w/v) sodium dodecyl sulfate (SDS), 0.1 *M* DTT, and 0.2% (w/v) bromophenol blue. Aliquot and store at –20°C.
3. Prestained molecular weight protein markers (#161-0305; Bio-Rad).
4. Acrylamide (30%):bisacrylamide (0.8%) solution (#161-0158; Bio-Rad).
5. Lower (separating) buffer: 1.5 *M* Tris-HCl, pH 8.8.
6. Upper (stacking) buffer: 0.5 *M* Tris-HCl, pH 6.8.

Table 2
Inhibitor of Initiators of MAPK Cascades

Inhibits[a]	Compound	Company (cat. no.)	Concentration used	Refs.
PI3K	LY-294002	BioMol (ST-420), Calbiochem (440202), Sigma (L-9908)	50 μM	*36*
	Wortmannin	BioMol (ST-415), Calbiochem (681675), Sigma (W-1628)	100 nM	*37,38*
PKC	GF 109203X	BioMol (EI-246), Calbiochem (203290), Sigma (B-6292)	2 μM	*39*
EGF-R	AG-1478	BioMol (EI-277), Calbiochem (658552), Sigma (T-4182)	0.25 μM	*40*
Src	PP1/PP2	BioMol (EI-275), Calbiochem (529579)	170 nM	*41*

[a]PI3K, phosphatidylinositol 3'-kinase; PKC, protein kinase C; EGF-R, epidermal growth factor receptor.

7. Tetramethylethylenediamine (#161-0800; Bio-Rad).
8. 10% ammonium persulfate (APS) (#161-0700; Bio-Rad).
9. Running buffer: 25 mM Tris; 192 mM glycine; 0.1% SDS, pH 8.3.
10. Staining solution: 40% methanol, 7% acetic acid, 0.005% bromophenol blue.
11. Destaining solution: 15% isopropanol, 7% acetic acid.

2.4. Western Blot Analysis

1. Transfer apparatus.
2. Transfer buffer: 15 mM Tris, 120 mM glycine, approximate pH of 8.8.
3. Nitrocellulose membrane (Protran BA 85; Schleicher & Schuell).
4. Whatman paper (3 mm).
5. Washing buffer (TBS-T): 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20.
6. Blocking solution: 2% (w/v) BSA in washing buffer.
7. Primary antibody appropriate for signaling MAPK of interest, such as monoclonal anti-diphospho-ERK (M-8159) and polyclonal anti-general ERK (M-5670) from Sigma Israel and secondary antibody (alkaline phosphatase [AP]- or horseradish peroxidase [HRP]-conjugated antimouse or antirabbit Fab antibodies from Jackson), diluted in washing buffer to appropriate dilutions.
8. Enhanced chemiluminescence (ECL): Commercial kits are available (Amersham; Pierce; Bio-Rad). Otherwise, ECL solutions can be made by mixing equal vol-

Table 3
Antibodies and Their Suppliers

Antibodies	Companies (cat. no.)
General ERK	Sigma (M 5670)
ERK1	Sigma (M 7927), Santa Cruz (Sc-93)
ERK2	Sigma (M 7556), Santa Cruz (Sc-154)
Phos-ERK	Sigma (M 8159), Santa Cruz (Sc-7383)
p38MAPK	Sigma (M 0800), Santa Cruz (Sc-535)
Phos-p38MAPK	Sigma (M 8177), Santa Cruz (Sc-7973)
JNK	Sigma (J 4500), Santa Cruz (Sc-474)
P-JNK	Sigma (J 4750), Santa Cruz (Sc-12882)
MEK	Sigma (M 5795), Santa Cruz (Sc-219)
p-MEK	Sigma (M 7683), Santa Cruz (Sc-7995)

umes of solution A (2.5 mM Luminol [#A-8511; Sigma], 400 µM p-coumaric acid [#C-9008; Sigma] in 100 mM Tris, pH 8.5) and solution B (5.4 mM H_2O_2 in 100 mM Tris-HCl, pH 8.5).

9. AP-based detection assay: nitrobluetetrazolium (NBT)/5-bromo-4-indolyl-phosphate (BCIP) visualization solution is comprises of 10 mL of AP substrate buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5.0 mM $MgCl_2$) containing 66 µL of NBT and 33 µL of BCIP (#S380C and #S381C; Promega)

2.5. Antibodies

See **Table 3** for antibodies.

2.6. Immunofluorescence

1. 6- or 12-well plates (Falcon).
2. 18-mm Cover slides (Marienfeld, Germany).
3. Paraformaldehyde.
4. Dye-conjugated secondary antibodies.
5. Mounting solution (Elvanol® or Mowiol 4-88).
6. Sandblasted frosted microscope slides (Chase).

3. Methods

3.1. Cell Culture

Cells are placed in growth medium (e.g., DMEM) supplemented with 10% heat-inactivated FCS, and cultured by heating it for 45 min at 56°C. In addition the medium contains 1% glutamine and an antibiotic mixture added to a final concentration of 100 U/mL of penicillin and 100 mg/mL of streptomycin. Adherent cells are periodically harvested with trypsin-EDTA from confluent

cultures. In experiments in which the goal is to test the phosphorylation state of specific proteins following stimulation or inhibition, the phosphorylation basal level should be reduced to minimum. This is achieved by incubating the cells in starvation medium (DMEM containing 0.1% FCS) 14–20 h prior to stimulation. The MAPKs are very sensitive to any alteration in the surrounding conditions; therefore, cells should not be removed from the incubator or handled in any other way at least 4 h before stimulation.

3.2. Detection of MAPK Inhibition

The inhibitors are added to the medium (for concentration and suppliers, *see* **Tables 1** and **2** and **Note 2**) for at least 15 min prior to the stimulant addition. The inhibition efficiency is often tested by comparing the MAPK activation to that in untreated cells. The phosphorylation rate of the MAPK members can be measured using phosphospecific antibodies. The advantage of these antibodies is that they bind to the MAPK proteins only when they are activated. Therefore, there is no need to perform an in vitro kinase assay for every sample. These antibodies can be purchased from commercial companies and can be used for immunoblotting or immunostaining (an example of immunoblotting appears in **Fig. 1**). The use of the antibodies in immunoblotting was described in Chapter 2, so here we only describe their usage in immunofluorescence studies. Commonly used antibodies are given in **Table 3**.

3.3. Immunofluorescence

The effect of the inhibitors on signaling pathways can be detected not only through immunoblotting but also by immunofluorescence. The advantage of immunofluorescence is the ability to observe the specific cellular localization of the protein of interest. Upon activation, the MAPKs, usually translocate to a deferent cellular compartment, especially to the nucleus.

1. Autoclave (dry) on 18-mm cover slide.
2. Add 18-mm cover slides to a 12- or 6-well dish, one slide in every well.
3. Harvest cells with trypsin-EDTA from cultures and add ~3×10^4 or 2×10^4 cells/ well to 6 or 12 wells, respectively.
4. After 24 h, incubate the cells with starvation medium for 14–20 h.
5. Add inhibitors + stimulant.
6. Wash cells with PBS twice.
7. Aspirate off the medium and add 3% formaldehyde/PBS (paraformaldehyde) to the cells (350–500 µL for every well) for 20 min at room temperature. Another fixation method is to incubate the cells with cold (–20°C) absolute methanol for 10 min at –20°C. *Optional:* the cells can be stored at 4°C for a few weeks.
8. Wash the cells with PBS three times, with an interval of 5 min between every wash. The rest of the procedure is at room temperature.
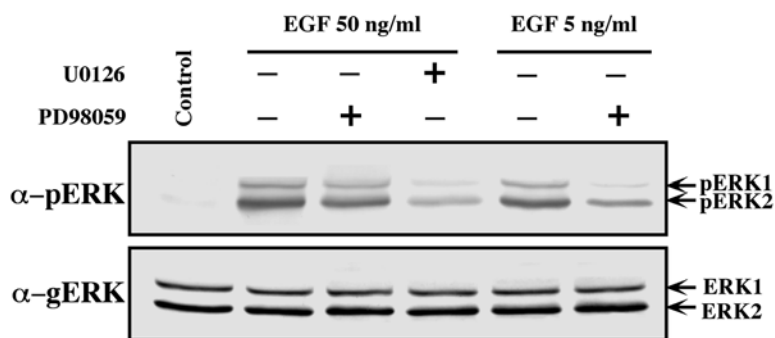
Fig. 1. The MEK1/2 inhibitors PD098059 and U0126 inhibit ERK1/2 and phosphorylation. HeLa cells were stimulated (or left unstimulated as control) with either 50 μ*M* or 5 μ*M* EGF for 7 min in the absence or presence of the MEK inhibitor PD098059 (25 μ*M*), and cells stimulated with 50 μ*M* EGF were inhibited with additional MEK1/2 inhibitor U0126 (5 μ*M*). Extracts (50 μg) were subjected to an sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with antibodies against the dual phosphorylation ERK (pERK) or against the general form of ERK (gERK).

9. For permeabilization, incubate the cells with 0.1% Triton X-100 in PBS (500 μL) for 2 min.
10. *Optional:* block with 2% BSA in PBS for 30 min (500 μL).
11. Incubate the primary antibody for 1 h. To save the antibody, spread out Parafilm and drop on it 40 μL of antibody for each slide. Remove the cover slides from the wells and place each one inverted on a pool of antibody. To prevent evaporation of the antibody, place the slides in a wet chamber.
12. Put the cover slides back into the wells and wash the cells three times with PBS.
13. Add the secondary antibodies in the same procedure as in **step 11**.
14. Repeat **step 12**.
15. Add 30 μL of evanol on the sandblasted frosted microscope slides and past the cover slide on the Elvanol (the cell side is toward the Elvanol).
16. *Optional:* Vacuum the remaining evanol and let it stand at room temperature until completely dry (a few hours). Fix the cover slide to the slide by adding nail polish around the cover slide.

4. Notes

1. 3-cyano-4-(phenoxyanilino)quinolines (from Wyeth-Ayerst) was identified by an in vitro screen for novel MEK inhibitors *(32)*. SL327, is a structural analogue of U0126 *(33)*. SB 2202190 (phenol, 4-[4-[4-fluorophenyl]-5-[4-pyridinyl]-1H-imidazol-2-y]) is a compound similar to the other p38 inhibitor SB 203580. Specificity test demonstrated that SB 220190 has a similar specificity to p38MAPK as SB 203580 *(16)*.

2. Data from knockout mice suggest that complete or even partial inhibition of the ERK pathway can have serious consequences. For example, targeted deletion of MEK1 is lethal owing to placental vasculature and fibroblast migration defects *(34)*. Knockouts, which do not completely inactivate the ERK transduction pathway (ERK1, RSK2), lead to defects in thymocyte maturation (ERK1), disorders of glycogen metabolism, and neurologic dysfunction such as Coffin-Lowry and Bardet-Biedl syndrome that occur owing to the absence or mutation of RSK2. We also found that nonselective ERK inhibition is not the best way to restore the balance in tumor cells. To develop a more effective inhibitor of ERK, it will be necessary to develop an agent that inhibits specific ERK1/2-mediated pathways involved in cell proliferation while leaving other pathways intact. Because inhibition of upstream effectors of ERK such as Raf-1 and MEK1/2 nonspecifically downregulate ERK1/2 activity, direct, selective inhibition of ERK will be required.

3. A few articles have appeared that describe the specificity and mode of actions of many of the inhibitors described here and of additional protein kinases. *See* **refs. 10**, **16**, **20**, and **35**.

References

1. Pearson, G., Robinson, F., Beers Gibson, T., et al. (2001) Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr. Rev.* **22,** 153–183.
2. Chen, Z., Gibson, T. B., Robinson, F., et al. (2001) MAP kinases. *Chem. Rev.* **101,** 2449–2476.
3. Seger, R. and Krebs, E. G. (1995) The MAPK signaling cascade. *FASEB J.* **9,** 726–735.
4. Abe, M. K., Kuo, W. L., Hershenson, M. B., and Rosner, M. R. (1999) Extracellular signal-regulated kinase 7 (ERK7), a novel ERK with a C-terminal domain that regulates its activity, its cellular localization, and cell growth. *Mol. Cell. Biol.* **19,** 1301–1312.
5. Abe, M. K., Saelzler, M. P., Espinosa, R. 3rd, et al. (2002) ERK8, a new member of the mitogen-activated protein kinase family. *J. Biol. Chem.* **277,** 16,733–16,743.
6. Wolf, I. and Seger, R. (2002) The mitogen-activated protein kinase signaling cascade: from bench to bedside. *Isr. Med. Assoc. J.* **4,** 641–647.
7. Hilger, R. A., Scheulen, M. E., and Strumberg, D. (2002) The Ras-Raf-MEK-ERK pathway in the treatment of cancer. *Onkologie* **25,** 511–518.
8. Sebolt-Leopold, J. S. (2000) Development of anticancer drugs targeting the MAP kinase pathway. *Oncogene* **19,** 6594–6599.
9. Gietema, J. A. and de Vries, E. G. (2002) Clinical cancer research 2002: new agents and therapies. *Drug Resist. Update* **5,** 192–203.
10. Dancey, J. and Sausville, E. A. (2003) Issues and progress with protein kinase inhibitors for cancer treatment. *Nature Rev.* **2,** 296–310.
11. Pages, G., Lenormand, P., L'Allemain, G., et al. (1993) Mitogen-activated protein kinases p42mapk and p44mapk are required for fibroblast proliferation. *Proc. Natl. Acad. Sci. USA* **90,** 8319–8323.

12. Seger, R., Biener, Y., Feinstein, R., Hanoch, T., Gazit, A., and Zick, Y. (1995) Differential activation of mitogen-activated protein kinase and S6 kinase signaling pathways by 12-O-tetradecanoylphorbol-13-acetate (TPA) and insulin. Evidence for involvement of a TPA-stimulated protein-tyrosine kinase. *J. Biol. Chem.* **270,** 28,325–28,330.

13. Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Saltiel, A. R. (1995) A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl. Acad. Sci. USA* **92,** 7686–7689.

14. Kamakura, S., Moriguchi, T., and Nishida, E. (1999) Activation of the protein kinase ERK5/BMK1 by receptor tyrosine kinases. identification and characterization of a signaling pathway to the nucleus. *J. Biol. Chem.* **274,** 26,563–26,571.

15. Favata, M. F., Horiuchi, K. Y., Manos, E. J., et al. (1998) Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J. Biol. Chem.* **273,** 18,623–18,632.

16. Davies, S. P., Reddy, H., Caivano, M., and Cohen, P. (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* **351,** 95–105.

17. Sebolt-Leopold, J. S., Dudley, D. T., Herrera, R., et al. (1999) Blockade of the MAP kinase pathway suppresses growth of colon tumors in-vivo. *Nat. Med.* **5,** 810–816.

18. Lyons, J. F., Wilhelm, S., Hibner, B., and Bollag, G. (2001) Discovery of a novel Raf kinase inhibitor. *Endocr. Relat. Cancer* **8,** 219–225.

19. Cuenda, A., Rouse, J., Doza, Y. N., et al. (1995) SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.* **364,** 229–233.

20. English, J. M. and Cobb, M. H. (2002) Pharmacological inhibitors of MAPK pathways. *Trends Pharmacol. Sci.* **23,** 40–45.

21. Lee, J. C., Laydon, J. T., McDonnell, P. C., et al. (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature* **372,** 739–746.

22. Johnson, G. L. and Lapadat, R. (2002) Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* **298,** 1911–1912.

23. Young, P. R., McLaughlin, M. M., Kumar, S., et al. (1997) Pyridinyl imidazole inhibitors of p38 mitogen-activated protein kinase bind in the ATP site. *J. Biol. Chem.* **272,** 12,116–12,121.

24. Eyers, P. A., Craxton, M., Morrice, N., Cohen, P., and Goedert, M. (1998) Conversion of SB 203580-insensitive MAP kinase family members to drug-sensitive forms by a single amino-acid substitution. *Chem. Biol.* **5,** 321–328.

25. Daum, G., Regenass, S., Sap, J., Schlessinger, J., and Fischer, E. H. (1994) Multiple forms of the human tyrosine phosphatase RPTP alpha. Isozymes and differences in glycosylation. *J. Biol. Chem.* **269,** 10,524–10,528.

26. Birkenkamp, K. U., Tuyt, L. M., Lummen, C., Wierenga, A. T., Kruijer, W., and Vellenga, E. (2000) The p38 MAP kinase inhibitor SB203580 enhances nuclear factor-kappa B transcriptional activity by a non-specific effect upon the ERK pathway. *Br. J. Pharmacol.* **131,** 99–107.

27. Haddad, J. J. (2001) VX-745. Vertex pharmaceuticals. *Curr. Opin. Invest. Drugs* **2,** 1070–1076.

28. Bennett, B. L., Sasaki, D. T., Murray, B. W., et al. (2001) SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc. Natl. Acad. Sci. USA* **98,** 13,681–13,686.

29. Glicksman, M. A., Chiu, A. Y., Dionne, C. A., et al. (1998) CEP-1347/KT7515 prevents motor neuronal programmed cell death and injury-induced dedifferentiation in-vivo. *J. Neurobiol.* **35,** 361–370.

30. Maroney, A. C., Finn, J. P., Connors, T. J., et al. (2001) Cep-1347 (KT7515), a semisynthetic inhibitor of the mixed lineage kinase family. *J. Biol. Chem.* **276,** 25,302–25,308.

31. Roux, P. P., Dorval, G., Boudreau, M., et al. (2002) K252a and CEP1347 are neuroprotective compounds that inhibit mixed-lineage kinase-3 and induce activation of Akt and ERK. *J. Biol. Chem.* **277,** 49,473–49,480.

32. Zhang, N., Wu, B., Powell, D., et al. (2000) Synthesis and structure-activity relationships of 3-cyano-4-(phenoxyanilino)quinolines as MEK (MAPKK) inhibitors. *Bioorg. Med. Chem. Lett.* **10,** 2825–2828.

33. Atkins, C. M., Selcher, J. C., Petraitis, J. J., Trzaskos, J. M., and Sweatt, J. D. (1998) The MAPK cascade is required for mammalian associative learning. *Nat. Neurosci.* **1,** 602–609.

34. Chang, L. and Karin, M. (2001) Mammalian MAP kinase signalling cascades. *Nature* **410,** 37–40.

35. Ahn, N. G., Nahreini, T. S., Tolwinski, N. S., and Resing, K. A. (2001) Pharmacologic inhibitors of MKK1 and MKK2. *Methods Enzymol.* **332,** 417–431.

36. Vlahos, C., Matter, W., Hui, K., and Brown, R. (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.* **269,** 5241–5248.

37. Arcaro, A. and Wymann, M. P. (1993) Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. *Biochem. J.* **296,** 297–301.

38. Wymann, M. P., Bulgarelli-Leva, G., Zvelebil, M. J., et al. (1996) Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. *Mol. Cell. Biol.* **16,** 1722–1733.

39. Toullec, D., Pianetti, P., Coste, H., et al. (1991) The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J. Biol. Chem.* **266,** 15,771–15,781.

40. Osherov, N. and Levitzki, A. (1994) Epidermal-growth-factor-dependent activation of the src-family kinases. *Eur. J. Biochem.* **225,** 1047–1053.

41. Hanke, J. H., Gardner, J. P., Dow, R. L., et al. (1996) Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. *J. Biol. Chem.* **271,** 695–701.

9

Structure of MAPKs

Elizabeth J. Goldsmith, Melanie H. Cobb, and Chung-I Chang

1. Introduction

Mitogen-activated protein kinases (MAPKs) are protein-serine/threonine kinases activated by signaling pathways triggered by developmental stages, cell-surface receptors, cell stresses and other environmental cues. The MAPK family includes the extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) and a splice variant of each, at least two ERK3 isoforms, ERK5, ERK7, four p38 MAP kinases (p38α, β, γ, and δ), and three c-Jun-N-terminal kinases/stress-activated protein kinases (JNK1–3/SAPKα, β, and γ), each with multiple splice variants *(1,2)*. These kinases are often categorized based on their most efficacious activators, although all are regulated by numerous overlapping stimuli. ERK1/2 are major targets of Ras-dependent signals and are usually most strongly activated by growth factors and proliferative stimuli. The p38 MAPKs and the JNK/SAPKs are recognized as stress sensors and, in some cases, promote apoptosis. ERK5 is significantly activated by growth factors and stresses and does not fits easily into either of these categories. Signals that activate ERK3 and ERK7 have not been determined.

The MAPKs are regulated by protein kinase cascades; two to three protein kinases acting in a series converge to activate a MAPK. The specificity of the cascade is determined significantly by the direct activators of MAPKs, the MAPK/ERK kinases or MAPK kinases (MEKs, MKKs, or MAP2Ks). These dual-specificity kinases phosphorylate a limited number of MAPKs, often no more than two members of the family. MEKs phosphorylate MAPKs on two residues, a single tyrosine and a single threonine within a TXY motif in the phosphorylation lip (also known as the activation loop), a regulatory loop that extends out of the active site of the protein kinases. The intervening residue X, glutamic acid in ERK1, 2 and 5, glycine in the p38 MAPKs, and proline

in JNK/SAPKs, contributes only minimally to MEK specificity. MAPK conformation and extended regions of interaction determine the selectivity of MEKs for MAPKs. Although single phosphorylations of MAPKs may cause changes in activity, the major increases in activity (for ERK2 >10,000-fold) *(3)* require phosphorylation of both residues. Phosphorylation also impacts the subcellular localization and formation of complexes by these kinases *(4)*. MEKs are themselves activated by phosphorylation by a diverse group of kinases with little obviously in common other than the ability to phosphorylate the two activation loop phosphoacceptor sites of MEKs. Of more than 20 protein kinases with MEKK or MAP3K (MAPK kinase kinase) activity in vitro or by cotransfection, the physiologic relevance of relatively few has been determined. Data are most clear for Raf isoforms, the major regulators of MEK1 and 2 in the ERK1/2 MAPK cascade. A number of these MAP3Ks are themselves regulated by phosphorylation by an even less well-defined group of kinases referred to as MAP4Ks, reflecting their position upstream of the MAPKs.

Because of the importance of MAPKs as signaling molecules, their unique form of regulation, and their potential as drug targets, they have been the focus of structural studies by us, and by several groups in the drug industry. Here we collate information on expression systems and crystallization conditions used in these structural analyses. The structures of low-activity ERK2 *(5)*, low-activity p38α *(6–8)*, and low-activity JNK3 *(9)* have each been solved. In addition, the active structures of ERK2 *(10)* and p38γ *(11)* have been determined crystallographically. Several crystallographic studies of inhibitors soaked into crystals of p38 and ERK2 have been conducted *(12–14)*. Tong and colleagues *(8,15)* cocrystallized a pyridinyl-imidazole compound with p38 and, by so doing, obtained different crystals.

Here we present the methods used in these crystallographic studies to express, purify, and crystallize the MAPKs. We also describe the conformational polymorphism in the phosphorylation lip as it changes on phosphorylation and how it is different among the MAPKs.

2. Materials

Materials used for expression, purification, and crystallization of various MAPKs are summarized in **Tables 1** and **2**. Chemicals, except those noted, are from Sigma (St. Louis, MO) and are SigmaUltra or analytical grade. Here we describe the expression vectors and coding sequences used to prepare MAPKs for crystallographic analysis.

Table 1
Expression and Purification of MAPKs[a]

Protein	Coding region from protein	Vector and tag	Cells	Culture medium	Induction	Column 1	Column 2	Column 3
Rat ERK2 *(5)*	1–359 (full length)	NpT7-5 N-tagged AH_6	BL21(DE3)	TB 100 µg/mL ampicillin 30°C	0.4 mM IPTG at OD_{600} =1.0 4 h	Ni^2-NTA agarose (Qiagen) 20 mM Tris-HCl, pH 7.5 20–250 mM imidazole (Im) gradient	MonoQ 100 mM–1 M NaCl 20 mM Tris-HCl, pH 7.5 5 mM DTT	
Mouse p38α *(6)*	1–360 (full length)	pET 14b (Qiagen), N-tagged GS_2H_6 S_2G-LVPRGMSQERP	BL21(DE3)	TB 100 µg/mL ampicillin 30°C	No induction 16 h	Ni-NTA agarose 50 mM Tris-HCl, pH 7.9 500 mM NaCl with 5 mM Im (buffer A) Wash: 20 mM Im Bump: 250 mM Im (buffer B)	Mono Q 50 mM Tris-HCl, pH 7.4 2 mM DTT with 100 (buffer A)– 500 mM NaCl (buffer B)	Phenyl Superose 50 mM Tris-HCl, pH 7.4 2 M NaCl (buffer A) 2 M–20 mM NaCl (buffer B)
Human p38 *(7)* (CSBP2)	1–360	pVL1392 (baculo-virus transfer vector) and pET 15b MGS_2H_6 S_2G-LVPRGSHMLE	High-Five Insect cells (Invitrogen)	Exel-405 Protein-free medium (JRH Bioscience) 27°C	Multiplicity of infection = 5 72 h	Talon,Co^{2+} agarose (Clonetech) 50 mM Na_2HPO_4, pH 8.0 20 mM NaCl 10% glycerol 2 mM 2-ME 0–100 mM Im	After thrombin cleavage Q-Sepharose in 25 mM HEPES, pH 7.5 25 mM phospho-glycerate 5% glycerol 2 mM DTT 0–600 mM NaCl	Anti-phospho-tyrosine agarose (Sigma)

(continued)

Table 1 (*continued)*

Protein	Coding region from protein	Vector and tag	Cells	Culture medium	Induction	Column 1	Column 2	Column 3
ERK2 *(10)* (active)	1–359	NpT7-5 co expressed with R4F *(4)* AH$_6$	BL21(DE3)	100 µg/mL ampicillin 30°C	0.4 mM IPTG at OD$_{600}$ = 0.8 12 h	Ni-NTA agarose 50 mM Na$_2$HPO$_4$, pH 7.2 300 mM NaCl 20-200 mM Im	Mono Q 20 mM Tris-HCl, pH 7.6 1 mM DTT, 100–250 mM NaCl	Phenyl Superose 50 mM HEPES, pH 7.5 1 M NaCl on ice 1 M–20 mM NaCl
JNK3 *(9)*	M-G-S40-E402 (fragment)	pET 15b No tag	BL21(DE3)	LB 100 µg/mL carbenicillin 30°C	0.8 mM IPTG at OD$_{600}$ = 0.8 2 h	SP Sepharose (Pharmacia) 20 mM HEPES, pH 7.0 10% glycerol (v/v) 2 mM DTT	Solution clarified by centrifugation	
p38/CSBP2 + inhibitor *(8)*	1–360	pQE60 (Qiagen) N-tagged H$_6$	B834(DE3)-plysS	DLM medium Kao & Michayuk vitamins 37°C	1 mM IPTG at OD$_{600}$ = 0.6 10 h	DEAE 25 mM Tris-HCl, pH 7.4 5% glycerol 50–400 mM NaCl	S100 25 mM Tris-HCl, pH 7.4 5% glycerol 1 mM DTT 4°C	

[a]TB, terrific broth; LB, Luria broth; Im, imidazole; DTT; dithiothreitol; IPTG, isopropylthio-β-D-galactoside; 2-ME, 2-mercaptoethanol.

Table 2
Crystallization of MAPKs[a]

	A: Protein solution	B: Precip. solution	C: Setup	D: Crystals	E: Space group
Rat ERK2 **(5)**	6 mg/mL ERK2; 50 mM Tris-HCl, pH 7.5, 5 mM DTT, 0.1 mM PMSF inhibitors[b]	18% w/v PEG 8000 (Alltech), 200 mM $(NH_4)_2SO_4$, 50 mM MES, pH 6.0	40 μL sitting drop, no seeds, 21°C	Prisms (0.7 × 0.4 × 0.3 mm^3)	P2$_1$, a = 49.3, b = 71.4, c = 61.2, b = 109.7, 2.3Å
Mouse p38α **(6)**	10 mg/mL p38α; 25 mM HEPES, pH 7.4, 50 mM NaCl, 10 mM DTT, 1 mM EDTA inhibitors[b]	18% w/v PEG 8000 (Fluka), 200 mM Mg(OAc)$_2$, 100 mM HEPES, pH 7.0	10 μL hanging drop microseeding, 20°C, 2 wk	Prisms (0.5 × 0.25 × 0.05 mm^3)	P2$_1$2$_1$2$_1$, a = 46.8, b = 84.9, c = 123.9, 1.8 Å
Human p38 **(7)** (CSBP2)	40 mg/mL CSBP2, 50 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM DTT, 5% glycerol	50 mM HEPES, pH 7.5, 560 mM sodium citrate, 130 mM $(NH_4)_2SO_4$	3 μL protein, 2 μL precip., vapor diffusion, room temperature, 3 d	Plates (0.35 × 0.25 × 0.03 mm)	P2$_1$2$_1$2$_1$, a = 45.2, b = 86.2, c = 123.9, 2.3 Å
ERK2-P2 **(10)** (active)	8 mg/mL ERK2-P2; 10 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM DTT, 0.1 mM EDTA, 0.05% (w/v) n-OG	20% w/v PEG 4000 (Fluka), 100 mM $(NH_4)_2SO_4$, 100 mM MES, pH 6.5	5 μL hanging, 3 d	Needles (0.3 × 0.06 × 0.06 mm^3)	P4$_1$2$_1$2, a = b = 92.5, c = 103.0, 2.4 Å

131

Table 2 *(continued)*

	A: Protein solution	B: Precip. solution	C: Setup	D: Crystals	E: Space group
p38α/pepMKK3b: SKGKSKRKKD-LRISCNSK (Chang et al., submitted)	8 mg/mL p38α; 3X peptide (molar ratio); 25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA	20% (w/v) PEG 8000 (Fluka), 100 mM Na cacodylate, pH 7.0, 100 mM Ca(OAc)$_2$	1 μL, 16°C, 3 d	Hollow rods (0.5 × 0.05 × 0.05 mm^3)	P3$_1$21, a = 81.9, b = 123.0, 2.3 Å
p38γ-P2 *(11)* (active)	21 mg/mL p38γ-P2, 5 mM AMP-PNP, 0.02% C$_{12}$E$_9$	2.7% (w/v) PEG 4000, 100 mM Tris-HCl, pH 8.5, 10 mM MgCl$_2$, 5 mM DTT	Vapor diffusion, 3–7 d	Clusters (0.1-mm thick)	P2$_1$2$_1$2$_1$, a = 63.5, b = 66.82, c = 206, 2.4 Å
JNK3 *(9)*	40 mg/mL JNK3; 2 mM MgCl$_2$, 1 mM AMP-PNP, 25 mM HEPES, pH 7.0, 5% glycerol (v/v), 50 mM NaCl, 10 mM DTT	20% (w/v) PEG 550, 10% (v/v) ethylene glycol, 20 mM 2–ME, 100 mM HEPES, pH 7.5	Hanging drop	Not specified	P2$_1$2$_1$2$_1$, a = 51.5, b = 71.2, c = 107.6, 2.3 Å
p38/CSBP2 + inhibitor *(8)*	6 mg/mL p38, 1.4 mM SB203580, 2.5 mM Tris-HCl, pH 7.4, 5% (v/v) glycerol, 50 mM NaCl, 1 mM DTT	30% (w/v) PEG 1500 (Fluka), 14 mM MgCl$_2$, 5 mM DTT, 1.4 mM AMPPNP	Vapor diffusion, 23°C, 2 wk	Prisms (0.7 × 0.15 × 0.15 mm^3)	P2$_1$2$_1$2$_1$, a = 65.2, b = 74.6, c = 78.1, 2.0Å

[a]DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; n-OG, n-octylglucoside; MES, morpholino ethanesulfonic acid; 2-ME, 2-mercaptoethanol; AMPPNP, adenyl β, γ−imidodiphosphate.

[b]Inhibitors of proteases: 1 μg of aprotinin/mL, 2 μg of leupeptin/mL, 120 μg of benzamidine/mL, 2 μg of pepstatin/mL.

2.1. Expression Systems of Low-Activity MAPKs

MAPKs possess both N- and C-terminal extensions from the core protein kinase domain defined by CDK2 *(16)*. The extensions are folded onto the core, and together with the "MAPK insertion," a 38-residue insert in the C-terminal of the kinase defines the unique structures of MAPKs. Most crystallographic studies have been carried out on full-length MAPKs (**Table 1**). An exception is JNK3, for which sequences outside the core kinase domain were removed to improve solubility and reduce susceptibility to proteolysis. In general, the kinases have been expressed with an N-terminal His$_6$ tag for use in purification. The only exception to this, again, is JNK3. Although the vector pET15b (Qiagen) was used, the sequences encoding the His$_6$ tag and thrombin cleavage site in the vector were eliminated.

We used NpT7-5 *(17)* to express ERK2 for crystallization. This vector fuses the tag directly to the inserted gene without an intervening linker sequence. For mouse p38α we used pET14b, which introduces a thrombin cleavage site between the tag and the protein coding sequence, but the tag was not proteolyzed prior to crystallization. Wilson et al. used a similar vector, pET15b, but the His$_6$ tag was removed by thrombin treatment.

MAPKs can be expressed in *Escherichia coli* (**Table 1**); this is the method we used for ERK2 and p38. When effective, *E. coli* expression has the advantage that the background of unwanted phosphorylation is not great. The NpT7-5 vector we used contains an ampicillin resistance gene. The expression levels for low-activity ERK2 are about 5–10 mg of purified protein/L of cell culture, when bacteria are grown at 30°C. Commercially available pET14b, pET15b, and pQE60 (Qiagen) were used in the studies of low activity p38 and JNK3. Low-activity p38 also expresses very well, consistently producing in 8–10 mg of purified protein/Lod cultured cells.

Interestingly, all of the groups working on these proteins have used the His$_6$ tag. We carried out expression and purification trials with the parallel vector system developed by Sheffield et al. *(18)*. We found that maltose-binding protein fusion was useful in expressing JNK2 (unpublished results). In general, both maltose-binding protein and glutatione *S*-transferase fusion proteins express better than the His$_6$ tagged protein. However, much of the protein of interest is lost during protease treatment to remove the tag. Thus, the His$_6$-tagged protein produced the best final yield. Additionally, eliminating the protease incubation shortens the time required for purification, which may be helpful in obtaining crystals.

2.2. Doubly Phosphorylated MAPKs

Successful strategies for expression of phosphorylated MAPKs have been described in detail *(19,20)*. Expression may employ one or two plasmids

encoding two or three protein kinases transformed into the same bacteria. However, to obtain quantities of activated MAPKs sufficient for crystallization, the single-plasmid systems proved to be preferable. The single-plasmid system consists of a MAPK and a constitutively active form of the appropriate MEK, such as ERK2 and the active MEK1 mutant MEK1R4F. These are subcloned into the same plasmid, each with appropriate translation initiation sites. The two-plasmid system uses a MAPK, a wild-type form of the appropriate MEK, and, in a separate plasmid, the catalytic domain of MEKK1—MEKK1 has been used because it efficiently phosphorylates MEK1–4, 6, and 7. Two plasmids may be preferred if repeated subcloning would be required otherwise, such as, to screen a series of mutants of either. To maintain expression of both plasmids, a different selection should be used for each. Low to modest levels of expression of the MEKK and the MEK are sufficient for activation of large quantities of the MAPK. Greatest yields of phosphorylated kinases generally result if bacteria are transformed with the relevant plasmids at each use. Multiple colonies may then be tested and highest expressors selected for large-scale phosphoprotein production. This is especially important with the two-plasmid method, in which different ratios of the two plasmids should be tested to achieve the best transformation efficiency. In the case of large-scale expression from a single plasmid, it is important that the OD_{600} of the bacterial culture be maintained below 0.4, because phosphoprotein expression decreases above this culture density.

3. Methods

Cell growth conditions and purification procedures used to prepare crystallization-grade MAPKs can be found in **Table 1**. Here we describe the expression, purification and crystallization of low activity p38α in greater detail.

3.1. Cell Growth

The transformed *E. coli* are grown in 2.5-L flasks in temperature-controlled incubators with an orbital-shaking platform. Even though cells can be grown to a higher density using a fermentor, our experience is that a better yield of p38α (and other MAPKs) per liter of cell culture can only be obtained from flasks.

1. Streak out *E. coli* strain BL21(DE3) that has been transformed with a vector encoding murine p38α in pET-14b (Novagen) on a fresh Luria-Bertani (LB) plate containing 100 µg/mL of ampicillin.
2. Incubate the plate overnight at 37°C for 16 h to obtain individual colonies.
3. Using a single colony, inoculate 10 mL of LB medium containing 100 µg/mL of ampicillin in a 25-mL flask, and grow the cells at 37°C while shaking at 300 rpm until the OD at 600 nm is about 0.6 (takes about 4 h) (*see* **Note 1**).
4. Use 500 µL of the midlog phase culture to inoculate 1 L of LB medium, 100 µg/mL of ampicillin (*see* **Note 2**). Grow the cells for 16 h at 30°C (*see* **Note 3**).

5. Harvest the cells by centrifugation using a Beckman J6-MI centrifuge with a 6 × 1 L swing-out rotor JS-4.2 for 40 min at 1500g and 4°C.
6. Remove the supernatant carefully and resuspend the cell pellet by vortexing in 50 mL of chilled Ni-buffer (*see* **Table 1**), and protease inhibitors (*see* **Table 2**). The cells either can be used immediately for purification of p38α or can be flash frozen in liquid N_2 and stored in a –80°C freezer.

3.2. Purification of MAPK p38α

3.2.1. Preparation of the Cell-Free Extract

1. Thaw the flash-frozen cells on ice.
2. Break the cells mechanically by passing the cell suspension twice through a French Press at 15,000–18,000 psi and 4°C. The change of the suspension from viscous to clear indicates that the cells have been completely broken.
3. Remove the cell debris by centrifuging for 1 h at 100,000g and 4°C in a Beckman Ti-45 rotor.
4. Transfer the cleared extract to a prechilled beaker and keep on ice.

3.2.2. Immobilized Metal Affinity Chromatography

1. Apply the extract from a 2-L culture at a flow rate of about 1.0 mL/min by using a peristaltic pump attached to an 8-mL Ni-NTA agarose (Qiagen) column, preequilibrated with 80 mL of Ni-buffer A (*see* **Table 1**).
2. After washing the column with at least 10 column volumes of wash (94% buffer A, 6% buffer B) (*see* **Table 1**), elute p38α with Ni-buffer B.
3. Collect fractions of 2 mL each. The elution takes about 20 min.
4. Pool the elution peak fractions, and dialyze the protein sample against 1 L of MonoQ buffer containing 100 mM NaCl (*see* **Table 1**) for 2 h. Change the buffer at least one time.

3.2.3. MonoQ HR Chromatography

1. Clear the dialyzed protein solution by passing it through a 0.22-µm filtered syringe.
2. Load the filtrate onto a MonoQ anion-exchange column (Pharmacia, Uppsala, Sweden) at 1.0 mL/min. The size of the column (either 5/5 or 10/10) is determined based on the amount of protein to be loaded (*see* **Note 4**).
3. After washing the column with 3 column volumes of MonoQ buffer A (*see* **Table 1**), apply an 80-mL gradient of 100–500 mM NaCl and collect 1.5-mL fractions.
4. Identify fractions containing p38α by 12.5% sodium dodecyl sufate-polyacrylamide gel electrophoresis (SDS-PAGE) using the PHAST system (Pharmacia). Generally, three peaks can be found in the chromatogram. The first peak, coming off at about 200 mM NaCl, is broad and contains the purest p38α. Fractions within this peak should be pooled and subjected to further purification.

3.2.4. Phenyl Superose HR Chromatography

Phenyl Superose chromatography is carried out at 4°C in 2 M NaCl, near the solubility limit for p38.

1. Slowly add 1× volume of 4 *M* NaCl (prechilled) to the protein solution on ice. The salt should be added very slowly, drop by drop, with great care to avoid precipitation

2. Clear the salt-adjusted protein solution by passing it through a syringe with a 0.22-μm filter attached.

3. Load the filtrate onto a 5/5 phenyl Superose column that has been equilibrated in PS-buffer A (*see* **Table 1**) at about 1.0 mL/min.

4. Wash the column with three column volumes of buffer A, until the OD_{280} is 0.

5. Elute the protein with an 80-mL gradient of 2 *M*–20 m*M* NaCl, and collect the fractions at 1.5 ml each.

6. Pool the peak fractions that contain pure p38α, and dialyze twice against 1 L of buffer of 50 m*M* HEPES (pH 6.8), 50 m*M* NaCl, and 4 m*M* dithiothreitol (DTT) (MonoS buffer), 2 h each.

3.2.5. MonoS HR Chromatography

p38α obtained after phenyl Superose chromatography is very (>99%) pure, as assessed by silver staining of an SDS-PAGE gel. However, the protein appears as a smear band on an isoelectric focusing gel. We found that further purification by passing the protein solution through a MonoS column significantly improves the homogeneity of p38α on an isoelectric focusing gel.

1. Clear the dialyzed protein solution by passing it through a 0.22-μm filtered syringe.

2. Apply the filtrate onto a 5/5 MonoS column equilibrated in MonoS buffer. Collect the flow-through fractions of 2 mL each.

3. Pool the flow-through fractions, and dialyze the protein solution against the storage buffer (*see* **Table 2, column A**).

3.2.6. Crystallization of MAPKs

We routinely use hanging-drop vapor diffusion techniques *(21)* for crystallization of p38α as well as other MAPKs. Linbro boxes, normally used for tissue culture, are commercially available (VDX plates from Hampton Research, Laguna Miguel, CA) for setting up crystallization experiments in hanging drops.

1. p38α in the storage buffer is concentrated using Centriprep (15 mL) and Centricon (2 mL) with 10-kDa cutoff membranes (Amicon) to 10 mg/mL at 4°C.

2. Clear the concentrated protein solution by centrifuging for 15 min at 13,000*g* at 4°C.

3. Grease the rims of the VDX plates with silicone grease.

4. Fill up the reservoirs with 0.6 mL of crystallizing agents (*see* **Table 2** and **Note 5**), which contain 18–20% PEG8000, 0.1 *M* HEPES (pH 6.8–7.1), and 0.2 *M* Mg(OAc)$_2$.

5. Mix a 1- to 5-μL drop of protein solution with an equal volume of reservoir solution.

6. Layer the drop on a 22-μm-thick cover slide (*see* **Note 6**).

7. Carefully turn over the cover slide and set it on the greased rim to allow the drop to be suspended over the well. Press gently to seal the well.

3.2.7. Microseeding and Cryoprotection

Initial p38α crystals appear sporadically in a week as small prisms. Microseeding is done to produce large p38α crystals.

1. Transfer one or more crystals onto a clean cover slide with a fresh drop of 5-μL reservoir solution.
2. Crush the crystals by overlaying a second cover slide onto the first cover slide and pushing the second slide against the first. Observe the crystals being crushed via a microscope.
3. Wash the crushed seeds by adding 50 μL of reservoir solution onto the surface of the first cover slide. Collect and transfer the solution from the cover slide to a microcentrifuge tube. This is the seed stock.
4. Dilute the seeds with the same reservoir buffer in the range of 10^{-3}–10^{-6} in a series of tubes. Vortex the tubes to evenly distribute the seeds.
5. Set up plates by mixing a 3-μL drop of protein solution with 3 μL of reservoir solution plus 0.3 μL of seed solution with a series of dilutions. Crystals appear in 3 d.
6. Cryoprotect cystals at a final concentration of 35% PEG 400 added to mother liquor by seven transfers to successively more concentrated PEG 400 solutions.

3.3. Solving MAPK Structures

The first MAPK structure, of low activity ERK2, was solved by multiple isomorphous replacement. Coordinates for cyclic adenosine monophosphate-dependent protein kinase (cAPK) were available *(22)*. However, molecular replacement failed, presumably because of the low sequence identity (23%), and also because of differences in the orientation of the two domains, and other differences in the N- and C-terminal extensions from the kinase core. Subsequent MAPK structure determinations have been carried out primarily by molecular replacement using either AmoRe *(23)*, XPLOR *(24)*, or CNS *(25)*. In determining the structure of p38, Wilson et al. *(7)* positioned the Patterson correlation refinement with the two domains of ERK2 as separate entities to obtain a model. We solved the structure similarly: the best molecular replacement solution revealed good electron density for the C-terminal domain, but very poor density for the N-terminal domain. Then we separated the N-terminal domain and docked it into the poor density in real space. Tong et al. *(15)* used anomalous dispersion data *(26)* based on selenomethionine but used molecular replacement to obtain initial phases. These phases were used to determine the selenomethionine positions. There are probably now a sufficient number of crystal structures that future structures can be solved by molecular replacement. It is never clear which molecule will provide the best search model. Interestingly, the low-activity JNK3 structure was solved based on molecular replacement using ERK2-P2.

3.4. The Lips of MAPKs

The phosphorylation lips, or activation loops, of MAPKs house the Thr-X-Tyr dual phosphorylation motif common to most MAPKs. In comparing the MAPK structures, the lip has emerged as hypervariable. It adopts a unique structure and forms unique interactions in each of the MAPKs, and it also changes dramatically when phosphorylated.

The lip is in the C-terminal domain, and the region that is variable extends from the glycine residue in the conserved DFG box, Gly167 in ERK2, to Arg189, one of the ligands for phosphotyrosine *(5)*. The structure of the lip in low-activity ERK2 is the most interesting. It makes all of its contacts to residues in the C-terminal domain and is found wrapped around the side-chains of two residues, Lys201 and Tyr203, in a neighboring loop (**Fig. 1A**) *(27)*. Phe181 and Leu182 of the lip make a close contact between helix G and the MAPK insert, touching Leu232 in helix G, and Tyr261 in the insert.

In low-activity ERK2 the P+1 specificity pocket is subtly blocked by the orientation of Val186 (side chain not shown) and Ala187, residues that line the P+1 site in analogy to cAPK *(28)*. Thr188, an important catalytic residue *(29)*, is in nearly the same position it has in the active structure.

In p38, the phosphorylation lip extends from Leu171 (168) to Arg186 (189) (ERK2 numbering in parentheses), six residues shorter than in ERK2 (**Fig. 1B**). Like ERK2, it forms contacts primarily in the C-terminal domain. However, the structure and locus of interaction is completely different. Residues Val183 to Arg186 are formed into a distorted helical turn and make a short helix with residues Arg186 to Arg189. This extension of the helix completely blocks the active site, moving the catalytic residue Thr185 (188) and the residues that form the P+1 site. In p38, the phosphorylation motif is Thr-Gly-Tyr. The glycine residue, Gly181 (not shown), forms an aromatic stacking interaction with the backbone of Thr226 in the loop between helices F and G (L13). Unlike ERK2, the phosphorylation lip of p38 makes some contacts in the N-terminal domain where Leu171 is in contact with Arg70 in helix C, and Ala172 falls between two arginine residues (Arg67 and Arg70) that are phosphothreonine ligands in the active structure *(11)*, and thus the phosphothreonine binding site is destroyed. Residues Arg173 to Asp177 are partially disordered. Also interesting in this region of the structure, Ile52 and Ile53 at the beginning of helix C are on the surface of the molecule, creating a hydrophobic patch (side chain not shown). The possible function of this patch is not known. In sum, the lip makes a few contacts in the N-terminal domain, is disordered, and then is folded up into a helical turn at its C-terminus, making contact in the C-terminal domain. There is nothing subtle in how the active site is destroyed in the low-activity form of p38, with the P+1 site, the P0 site at the active site, and the phosphothreonine binding sites all missing in this structure.
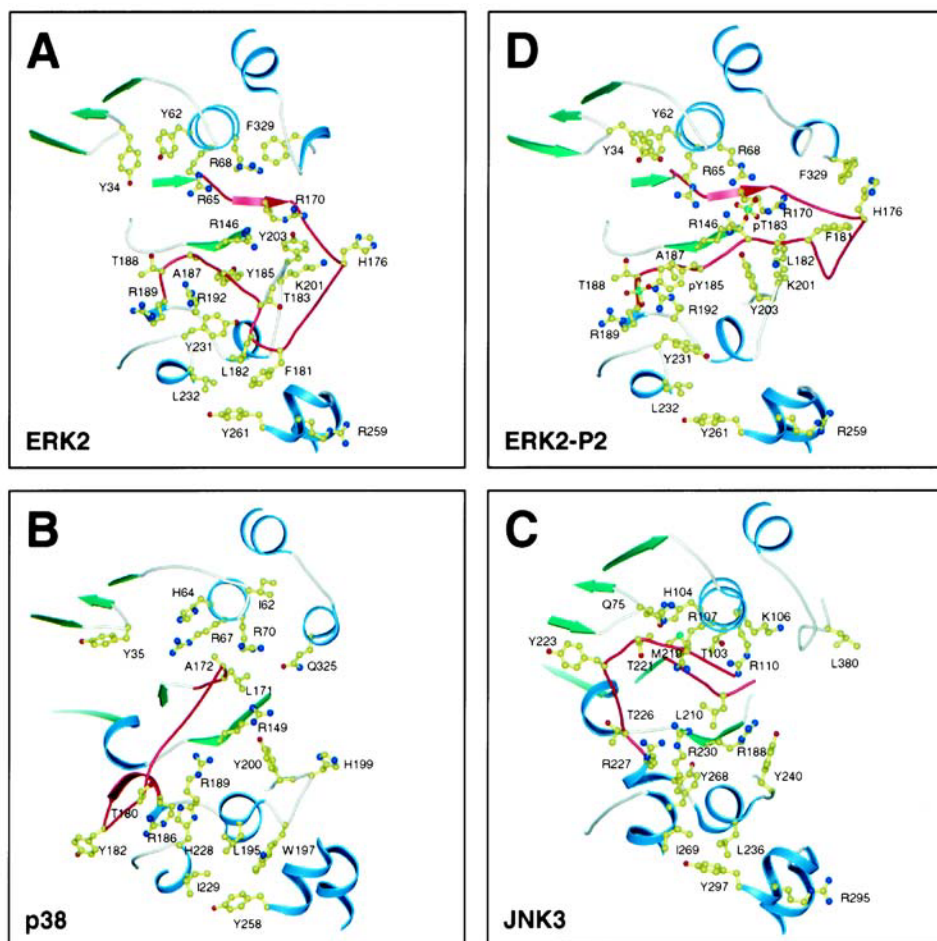
Fig. 1. Interaction between phosphorylation lip and neighboring structures in **(A)** ERK2, **(B)** ERK2-P2, **(C)** p38, and **(D)** JNK3 (PDB file 1JNK). Prepared with Bobscript *(30)*, gl_render (L. Esser, personal communication) and POV-Ray (Persistence of Vision Ray Tracer v3.1g, © 1999 POV-Team).

In JNK3, the lip spans residues Leu210–Arg227 and includes the JNK3 regulatory phosphorylation sites Thr221 (183) and Tyr223 (184) in the sequence Thr-Pro-Tyr. The lip is four residues shorter than in ERK2. In contrast to ERK2 and p38, it makes its contacts exclusively in the N-terminal domain, and with itself (**Fig. 1C**). The lip is folded over the phosphate binding ribbon, and Met219 makes contacts with Thr103 (61) and His104 (Tyr62) at the N-terminus of helix C. Gly209 (167) of the DFG sequence and Leu210 (168) (side chain not shown) bind between the phosphothreonine ligands Arg107 (65) and

Arg110 in a fashion somewhat analogous to low-activity p38. As in p38, the phosphothreonine binding site is blocked by this interaction. The active site is massively destroyed otherwise, with the catalytic Thr226 (188) in a different position, and the P+1 site not formed. Like p38, part of the lip is disordered.

The lip changes entirely on activation (**Fig. 1D**). In the active form of ERK2, most of the interactions in the C-terminal domain are lost, in favor of new interactions in the N-terminal domain. A new stacking interaction is formed between His-176 in the lip and Phe-329 (in L16) in the N-terminal domain. The contacts in the N-terminal domain are completely different from those formed by JNK3, being primarily with L16 rather than with the phosphate-binding ribbon as with JNK3. In the refolding, Phe-181 and Leu-182, which form a strong tether to the C-terminal domain in the low-activity structure, are participating in the new fold of the lip (Phe-181) or are on the surface (Leu-182). The pocket between helix G and the MAPK insert occupied by Phe181 and Leu182 in the low-activity structure is empty in ERK2-P2. Thus, new interactions are formed and old ones are lost. The lip is also no longer wrapped around Lys-201 and Tyr-203 but, instead, makes fewer contacts with these residues and becomes threaded between them. In the active form of p38γ, the interactions of the phosphothreonine and phosphotyrosine are very similar to those of ERK2-P2 *(11)*, but the lip is shorter, and missing the interactions with L16 present in ERK2 (not shown). There is no strong refolding of L16 in p38γ -P2. In p38, the helical turn beginning at Phe329 (Gln325 in p38), which forms on activation in ERK2, is already present in the low-activity structure, at least in p38α (**Fig. 1B**).

In comparing these structures, it is interesting to note that the lips each form distinct interactions: ERK2 interacts entirely with the C-terminal domain, and p38 interacts with the N-terminal domain, is disordered, and then interacts extensively with the C-terminus. In JNK, the lip binds primarily to the phosphate-binding ribbon and helix C in the N-terminal domain. In active ERK2, the lip also binds the N-terminal domain, but differently, and completes the active site at the C-terminus by forming the P+1 site. The lip is a variable and adjustable linker. It is part of the C-terminal domain but bridges the two domains, modulating their separation, and either blocking or completing the active site of the kinase.

4. Notes

1. For the expression of other MAPKs that requires induction with IPTG, the OD_{600} of the culture should not go above 0.8 at the time of induction. Induction of recombinant protein expression in late-log phase of culture usually results in poor expression and high cellular protein background.
2. We prefer to use acid-stable carbenicillin rather than ampicillin in LB medium when growing overnight culture to ensure the maintenance of selection pressure.

3. For the expression of other MAPKs that requires IPTG induction, the induction time must be strictly followed. We have observed a decrease in protein yield in cultures incubated with IPTG overnight.
4. To achieve better separation result, we choose a column whose maximum binding capacity is at least two times larger than the amount of protein to be applied.
5. All reagents used for making crystallizing agents have to be filtered through a 0.22-μm membrane.
6. Siliconized glass cover slides, available from Hampton Research are preferred because their hydrophobic surface generally produces a drop that does not flatten on the glass.

References

1. Lewis, T. S., Shapiro, P. S., and Ahn, N. G. (1998) Signal transduction through MAP kinase cascades. *Adv. Cancer Res* **74,** 49–139.
2. Pearson, G., Robinson, F., Beers Gibson, T., et al. (2001) Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr. Rev.* **22,** 153–183.
3. Prowse, C. N., Hagopian, Jonathan C., et al. (2000) Catalytic reaction pathway for the mitogen-activated protein kinase ERK2. *Biochemistry* **39,** 6258–6266.
4. Khokhlatchev, A. V., Canagarajah, B., Atkinson, M., Goldsmith, E., and Cobb, M. H. (1997) Phosphorylation of the map kinase ERK2 promotes its homodimerization and nuclear translocation. *Cell* **93,** 605–615.
5. Zhang, F., Strand, A., Robbins, D., Cobb, M. H., and Goldsmith, E. J. (1994) Atomic structure of the MAP kinase ERK2 at 2. 3Å resolution. *Nature* **367,** 704–711.
6. Wang, Z., Harkins, P. C., Ulevitch, R. J., et al. (1997) The structure of the mitogen-activated protein kinase P38 at 2. 1 Å resolution. *Proc. Natl. Acad. Sci. USA* **94,** 2327–2332.
7. Wilson, K. P.,Fitzgibbon, M. J., Caron, P. R., et al. (1996) Crystal structure of the p38 mitogen-activated protein kinase. *J. Biol. Chem.* **271,** 27,696–27,700.
8. Pav, S., White, D. M., Rogers, S., et al. (1997) Crystallization and preliminary crystallographic analysis of recombinant human p38 MAP kinase. *Protein Sci.* **6,** 242–245.
9. Xie, X., Gu, Y, Fox, T., et al. (1998) Crystal structure of JNK3: a kinase implicated in neuronal apoptosis. *Structure* **6,** 983–991.
10. Canagarajah, B. J., Khokhlatchev, A., Cobb, M. H., and Goldsmith, E. J. (1997) Activation mechanism of the MAP kinase ERK2 by dual phosphorylation. *Cell* **90,** 859–869.
11. Bellon, S., Fitzgibbon, M. J., Fox, T., Hsiao, H.-M., and Wilson, K. P. (1999) The structure of phosphorylated P38γ is monomeric and reveals a conserved activation loop conformation. *Structure* **7,** 1057–1065.
12. Wilson, K. P., McCaffrey, P. G., Hsiao, K., et al. (1997) The structural basis for the specificity of pyridinylimidazole inhibitors of p38 MAP kinase. *Chem. Biol.* **4,** 423–431.

13. Wang, W., Canagarajah, B. J., Boehm, J. C., et al. (1998) Structural basis of inhibitor selectivity in MAP kinases. *Structure* **6,** 1117–1128.

14. Shewchuk, L., Hassell, A., Wisely, B., et al. (2000) Binding mode of the 4-anilinoquinazoline class of protein kinase inhibitor: x-ray crystallographic studies of 4-anilinoquinazolines bound to cyclin-dependent kinase 2 and p38 kinase. *J. Med. Chem.* **1,** 133–138.

15. Tong, L., Pav, S., White, D. M., et al. (1997) A highly specific inhibitor of human p38 MAP kinase binds in the ATP pocket. *Nat. Struct. Biol.* **4,** 311–316.

16. Hanks, S. K. and Hunter, T. (1995) The eukaryotic protein kinase super-family: kinase (catalytic) domain structure and classification. *FASEB J.* **9,** 576–596.

17. Graziano, M. P., Freissmuth, M., and Gilman, A. G. (1989) Expression of $G_{s\alpha}$ in *Escherichia coli*. *J. Biol. Chem.* **264,** 409–418.

18. Sheffield, P., Garrard, S., and Derewenda, Z. (1999) Overcoming Expression and Purification Problems of RhoGDI Using a Family of Parallel Expression Vectors. *Protein Expression Purification* **15**, 34-39.

19. Wilsbacher, J. L. and Cobb, M. H. (2001) Bacterial expression of activated MAP kinases. *Meth. Enzymol.* **332,** 387–400.

20. Khokhlatchev, A., Xu, S., English, J., et al. (1997) Reconstitution of mitogen-activated protein kinase phosphorylation cascades in bacteria: efficient synthesis of active protein kinases. *J. Biol. Chem.* **272,** 11,057–11,062.

21. Ducruix, A. and Giege, R. (1999) *Crystallization of Nucleic Acids and Proteins*, Oxford University Press, Oxford, UK.

22. Knighton, D. R., et al. (1991) Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* **253**, 407-413.

23. Navaza, J. E. (1992) *AMoRe: A New Package for Molecular Replacement,* SERC, Daresbury, UK.

24. Brunger, A. T. (1992) *X-PLOR Version 3.0: A System for Crystallography and NMR*, Yale University Press, New Haven, CT.

25. Brunger, A. T., Adams, P. D., Clore, G. M., et al. (1998) Crystallography and NMR system: a new software suite for macromolecular structure determination. *Acta Crystallogr. D Biol. Crystallogr.* **54,** 905–921.

26. Hendrickson, W. A., Smith, J. L., Phizackerley, R. P., and Merritt, E. A. (1997) Phase Determination from Multiwavelength Anomalous Diffraction Measurements. *Meth. Enzymol.* **276,** 494–523.

27. Zhang, J., Zhang, F., Ebert, D., Cobb, M. H., and Goldsmith, E. J. (1995) Activity of the MAP kinase ERK2 is controlled by a flexible surface loop. *Structure* **3,** 299–307.

28. Knighton, D. R., Zheng, J. H., Ten Eyck, L. F., et al. (1991) Structure of a peptide inhibitor bound to the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* **253,** 414–420.

29. Gibbs, C. S., Knighton, D. R., Sowadski, J. M., Taylor, S. S., and Zoller, M. J. (1992) Systematic mutational analysis of cAMP-dependent protein kinase identi-

fies unregulated catalytic subunits and defines regions important for the recognition of the regulatory subunit. *J. Biol. Chem.* **267,** 4806–4814.

30. Esnouf, R. M. (1999) Further additions to Mo1Script Version 1. 4, including reading and contouring of electron-density maps. *Acta Crystallogr. D Biol. Crystallogr* **55,** 938–940.

10

Subcellular Localization of MAPKs

Makoto Adachi and Eisuke Nishida

1. Introduction

Because there are several nuclear substrates for the members of the mitogen-activated protein kinase (MAPK) superfamily, they should become localized to the nucleus to achieve their functions. For example, whereas extracellular signal-regulated kinase (ERK) predominantly localizes to the cytoplasm in quiescent cells, it translocates to the nucleus upon its activation by mitogenic stimuli *(1–3)*. Nuclear localization of ERK is transient, however, and ERK is exported from the nucleus to the cytoplasm when it is inactivated. Similarly, ultraviolet irradiation and osmotic stimuli induce activation and transient nuclear localization of c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 MAPKs *(4–6)*. To adequately perform their functions, not only the activation state but also the subcellular localization must be tightly regulated.

Among MAPKs in higher eukaryotes, ERK is most extensively studied for its role in the regulatory mechanism of nucleocytoplasmic transport. Our current knowledge may be outlined as follows. In quiescent states ERK is retained to the cytoplasm by directly interacting with its cytoplasmic anchoring proteins such as MEK *(7)* through the C-terminal common docking domain of ERK *(8,9)*. Apparently, MEK always localizes to the cytoplasm because it has a nuclear export signal (NES) *(10)*. On phosphorylation by MEK, ERK dissociates from MEK *(7,11)*, and at least part of the phosphorylated ERK forms dimers with either phosphorylated or unphosphorylated ERK *(12)*. Dimerized ERK can pass through nuclear pores by utilizing an active transport mechanism, whereas monomeric ERK can also enter the nucleus by passive diffusion *(11)*. Nuclear accumulation of ERK requires an as-yet unidentified nuclear anchoring protein, whose synthesis is dependent on the activation of the ERK pathway *(13)*. When ERK is inactivated and the nuclear anchoring protein is

degraded, ERK is exported from the nucleus to the cytoplasm. MEK, which transiently enters the nucleus by diffusion, binds to nuclear ERK, and then the MEK-ERK complex is exported from the nucleus by an NES-dependent active transport mechanism *(14)*.

To understand the regulatory mechanism of the nucleocytoplasmic transport of ERK, several points still need to be addressed. First, it is not known what the nuclear anchor of ERK is (although the transcription factor Atf1 is reported to function as a nuclear anchor for the stress-activated MAPK Sty1/Spc1 in fission yeast *[15]*). Second, there could be another cytoplasmic anchor for ERK other than MEK, because ERK, in general, is more abundant than MEK in cells *(16)*. Third, how active nuclear transport of dimerized ERK is achieved is unclear. Because it is reported that active nuclear transport of dimerized ERK is dependent on Ran (a low molecular weight guanosine 5'-triphosphotase that is prerequisite for importin-dependent active transport mechanisms) *(11)* and that an importin 7 homolog DIM-7 is required for nuclear accumulation of D-ERK in *Drosophila (17)*, it is likely that dimerization of ERK induces its direct or indirect association with the importin family molecule. Studies are necessary to test this hypothesis.

Recently, Kim-Kaneyama et al. *(18)* and Lim et al. *(19)* reported that in senescent fibroblasts, activation of ERK by mitogenic stimuli is induced as strongly as that in presenescent cells, whereas nuclear accumulation of ERK is severely impaired. Similarly, Aplin et al. *(20)* reported that in nonadherent cells stimulus-dependent nuclear accumulation of ERK is impaired whereas activation of ERK occurs normally. Although it is unclear whether the failure of nuclear accumulation is caused by defects in nuclear transport mechanism or in nuclear accumulation, these results may imply the presence of a hitherto unknown regulatory step(s), which lies downstream of the ERK activation step, in the mechanism of nuclear translocation of ERK.

Thus, further investigations are necessary to understand fully the molecular mechanism of nucleocytoplasmic transport of ERK. Here we describe techniques that may be helpful for studying these aspects. They include methods for staining of ERK in cells and for detecting the binding of ERK to its interacting proteins. Essentially similar strategies may be applicable for the studies of other MAPKs such as JNK/SAPK and p38, and for studies of possible translocation of active ERK to cellular compartments other than nucleus, such as cell periphery *(2,21)*.

2. Materials

2.1. Detection of Subcellular Localization of ERK by Cell Staining

2.1.1. Cell Culture (see **Notes 1** and **2**)

1. Medium and serum suitable for the cell used.
2. One 35-mm dish or six-well plate.
3. Cover slip (18 × 18 mm).
4. CO_2 incubator.

2.1.2. Induction of Nuclear Translocation or Nuclear Export of ERK

1. Serum-free (or low-serum) medium.
2. Stimulus (i.e., epidermal growth factor [EGF], serum).

2.1.3. Cell Staining (see **Note 3**)

1. Formaldehyde (37 % [w/v]).
2. Phosphate-buffered saline (PBS): 137 m*M* NaCl, 8.1 m*M* Na_2HPO_4, 2.68 m*M* KCl, 1.47 m*M* KH_2PO_4 (pH 7.4).
3. 10% Triton X-100/PBS (v/v).
4. 3% Bovine serum albumin (BSA)/PBS: Dissolve 3% BSA in PBS (w/v). Store at –20°C.
5. Primary antibody.
6. Fluorescence-labeled secondary antibody.
7. Mowiol: Mix 2.4 g of Mowiol 4-88 (475904, Calbiochem), 6.0 g of glycerol, and 6.0 mL of ddH$_2$O for 2 h at room temperature. Add 12 mL of 0.2 *M* Tris-HCl (pH 8.5) and mix for 2 h to overnight at 50°C. Centrifuge at 5000*g* for 15 min. Protect from light and store at –20°C.
8. Rack for cover slip.
9. Cups.
10. Moist chamber: a plastic box on which a wet paper towel is laid. Be sure not to get the paper too wet; otherwise the antibody solution on the cover slip will be lost. Alternatively, lay a piece of Parafilm. In either case, put wet paper at the edge of the box to avoid drying the antibody solution.
11. Slide (76 × 26 mm).

2.2. Binding Assay

To detect the binding of ERK to its interacting proteins, here we present the coimmunoprecipitation assay, in which ERK and its binding partner are overexpressed in cells. Similar methods may be applicable for the detection of the binding between endogenous proteins.

2.2.1. Transfection

1. Mammalian expression vectors encoding ERK and its binding protein, tagged with different epitopes (e.g., myc-tagged ERK and HA-tagged MEK).
2. Transfection reagents (e.g., Lipofectamine from Life Technologies).
3. Cells and medium.
4. One 60-mm dish.
5. CO_2 incubator.

2.2.2. Binding Assay

1. Extract buffer (E buffer): 20 mM HEPES-NaOH (pH 7.4), 2 mM MgCl$_2$, 2 mM EGTA. Store at 4°C.
2. E* buffer: Add the following to E buffer just before use (each in final concentration): 1% aprotinin, 1 mM PhCH$_2$SO$_2$F, 2 mM dithiothreitol, 1 mM Na$_3$VO$_4$, and 1 mM NaF (*see* **Note 4**).
3. Antibody for the epitope tag.
4. Protein A-Sepharose (Pharmacia, Uppsala, Sweden).
5. Elution buffer: 100 mM Gly-HCl, pH 2.2. Store at 4°C.
6. Neutralization buffer: 1.5 M Tris-HCl, pH 8.8. Store at 4°C.
7. Laemmli's sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer.

3. Methods

3.1. Detection of Subcellular Localization of ERK by Cell Staining

3.1.1. Induction of Nuclear Translocation of ERK

All procedures described in this section and in the following section should be performed on a clean bench.

1. Sterilize a cover slip by dipping in 100% EtOH. Dry and put in a 35-mm dish.
2. Seed appropriate cells in the dish and culture overnight in a CO_2 incubator.
3. The next day (16–24 h later), change the medium to serum-free medium after washing once. For some types of cells (e.g., NIH3T3), use low-serum medium to keep the cells from dying.
4. Culture in a CO_2 incubator for 36–48 h to arrest the cells in a quiescent state (at G_0).
5. Add the stimulus (e.g., 30 nM EGF, 10% fetal calf serum [FCS], 100 ng/mL of TPA) to the culture medium to activate the ERK cascade. Incubate for 5 min to 6 h (*see* **Note 5**).

3.1.2. Induction of Nuclear Translocation or Nuclear Export of ERK

1. Induce the nuclear translocation by stimulus as described in **Subheading 3.1.1., step 5**.
2. Change the medium to serum-free medium after washing once.
3. Culture for 5 min to 2 h (*see* **Note 6**).

3.1.3. Cell Staining

1. Add formaldehyde to a final concentration of 3.7% in the culture medium.
2. Incubate for 10 min to1 h to fix.
3. Wash the cover slip twice with PBS (5 min each wash). Use cups and racks.
4. Permeabilize the cell in 0.5% Triton X-100/PBS for 5 min; to make 0.5% Triton X-100/PBS, dilute the 10% Triton X-100/PBS stock solution with PBS. Do not directly dilute the detergent, because Triton X-100 is so viscous that it is difficult to measure its volume correctly.
5. Wash twice with PBS (5 min each wash).
6. Wipe the cover slip by tapping its side on the filter paper. Put it in the moist chamber, and immediately mount 50 µL of the primary antibody solution (anti-ERK antibody diluted 1:100 with 3% BSA/PBS) (*see* **Note 7**).
7. Incubate for 1 to 2 h at room temperature or overnight at 4°C.
8. Wash with PBS twice, 5 min each wash.
9. Put the cover slip in the new moist chamber as in **step 6**, and then mount fluorescence-labeled secondary antibody solution (fluorescene isothiocyanatte– or rhodamine-iso-thio-cyanate (RITC)-conjugated antirabbit IgG [or equivalent] diluted with 3% BSA/PBS).
10. Incubate for 1 h at room temperature. Protect from light in the following procedures (wrap with aluminum foil or alternative).
11. Wash with PBS twice, 5 min each wash.
12. Put separately two drops of Mowiol on a slide. Do not leave it longer than a few minutes, or it will become dry.
13. Wash the cover slip with ddH$_2$O briefly, and then wipe it by tapping its side on the filter paper. Put it on a drop of Mowiol with its upside (to which cells adhere) down. Avoid including bubbles.
14. Dry for at least 1 h before observation.

3.2. Binding Assay

1. Seed appropriate cells in a 60-mm dish. The number of cells should be optimized to the condition of transfection (e.g., transfection by means of lipofection).
2. The next day, transfect the cells with the expression vectors encoding epitope-tagged ERK and the protein of interest. The concentration of each vector might be equal.
3. The following day, wash the cell twice with ice-cold E buffer.
4. Lyse the cells with 300 µL of E* buffer. Samples must be kept on ice throughout the following procedures.
5. Homogenize the cells with a rotating homogenizer (at 1200 rpm for 10 s, repeat three times) (*see* **Note 4**).
6. Centrifuge at 15,000*g* for 30 min at 4°C.
7. Keep 60 µL of the supernanant as whole cell extract. Mix the remainder (240 µL) with an antibody (for the epitope of either of the overexpressed proteins) and 50 µL of protein A-Sepharose beads.
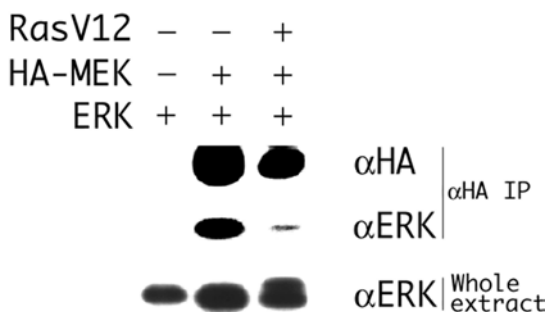
Fig. 1. Example of MEK-ERK binding assay. The indicated constructs were coexpressed in COS7 cells, and then cell lysates were subjected to immunoprecipitation with anti-HA antibody followed by immunoblotting with anti-ERK antibody. ERK coprecipitated well with HA-MEK in the absence of stimuli, whereas coexpression of RasV12, which stimulates the MEK-ERK cascade, resulted in a marked decrease in the coprecipitated ERK (αHA IP, αERK).

8. Incubate with a rotate circulator for 1.5 h at 4°C.
9. Wash the beads four times with 300 μL of E* buffer by inverting the tubes five to six times (*see* **Note 8**).
10. Add 300 μL of elution buffer and mix by tapping. Incubate for 1 min.
11. Centrifuge at 3500*g* for 1 min at 4°C.
12. Transfer 150 μL of an eluate to a new tube, in which 12 μL of neutralization buffer is added beforehand. Avoid contaminating the beads. Mix by tapping (*see* **Note 9**).
13. Add SDS-PAGE sample buffer to the whole-cell extract and the eluate, and then boil. Now it is ready for analysis by immunoblotting.

An example of the result of the binding assay is shown in **Fig. 1**.

4. Notes

1. As far as we have tested, stimulus-dependent nuclear translocation of ERK can be observed in many kinds of cultured cells, including NIH3T3, Swiss3T3, 3Y1, A6, and COS7 cells. Note, however, that there are differences in the extent and rapidity of nuclear accumulation of ERK. Moreover, nuclear accumulation does not always occur in sufficient populations. In these cases, lot selection or cloning of the cells may be necessary.
2. One may want to see the stimulus-dependent nuclear translocation of overexpressed ERK. However, this is often very hard. This is presumably because growth factor stimulation is not "strong" enough to activate a sufficient population of overexpressed ERK. One solution is to use a cell line such as ΔB-Raf:ER cells, in which B-Raf can be conditionally activated (or inactivated) by the addition (or removal) of estradiol *(22)*. Because of the strong kinase activity of B-Raf, this system allows us to activate nearly all overexpressed ERK within 5 min

of the stimulation *(11)*. Alternatively, coexpression with activated forms of MEK is also effective.

3. To detect the subcellular localization of endogenous ERK in mammalian cells, we use a rabbit anti-ERK polyclonal antibody (K-23) purchased from Santa Cruz Biotechnology, although other commercially available antibodies will also be applicable (e.g., anti-ERK1+ERK2 monoclonal antibody [MAb] from Zymed). There are also antibodies that detect only an activated (dually phosphorylated) form of ERK (E-10 monoclonal antibody from Cell Signaling Technology; M8159 MAb from Sigma, St. Louis, MO), and they also can be used for staining. However, we describe here methods for K-23 only. When using other antibodies, the protocol may need to be modified (e.g., there are alternative methods for fixation).

4. We did not include detergent or salt in E buffer, because they may interfere with the binding. However, we found that the addition of 1% NP-40 and/or 150 m*M* NaCl did not have a strong inhibitory effect. If NP-40 is added, the homogenization step is not required for cell lysis. Thus, step 5 in the binding assay is to be omitted, and do pipeting instead. Finally, the addition of 10% glycerol may help to stabilize the binding.

5. The speed of nuclear translocation of ERK differs depending on the cell or the stimulus used. For example, we found that FCS stimulation of NIH3T3, A6, or COS7 cells induced "slow" nuclear translocation of ERK, in which nuclear accumulation of ERK was observed 4–6 h after stimulation *(11)*. On the other hand, TPA stimulation of 3Y1 or COS7 cells induced "rapid" nuclear translocation, which occurred as fast as 5 min after stimulation *(11)*.

6. As is the case for nuclear translocation, the speed of nuclear export of ERK varies with the cell. In A6 cells, nuclear export is apparent 10 min after serum removal, whereas in NIH3T3 cells it is sometimes barely seen even after 2 h. In 3Y1 cells in which nuclear translocation is achieved 5 min after stimulation with TPA, nuclear export is clearly detected as fast as 10 min after stimulation, even if TPA is not removed from the culture medium *(14)*.

7. We could clearly stain the endogenous ERK without blocking in all cell lines tested. If desired, however, blocking with 3% BSA/PBS prior to the addition of the primary antibody for 1 h at room temperature may be effective.

8. Take great care with this step. Avoid intense wash.

9. If you want to detect the coimmunoprecipitated ERK in the ERK-interacting protein immunoprecipitate, this method (**Subheading 3.2, steps 10–12**) is prerequisite. ERK protein sometimes nonspecifically adsorbs to the protein A-Sepharose beads. Then, the elution of the protein by a conventional method (i.e., adding the sample buffer to the beads and then boiling) is not suitable. Alternatively, blocking the beads with BSA prior to immunoprecipitation may be suitable.

References

1. Chen, R.-H., Sarnecki, C., and Blenis, J. (1992) Nuclear localization and regulation of *erk-* and *rsk-*encoded protein kinases. *Mol. Cell. Biol.* **12,** 915–927.

2. Gonzalez, F. A., Seth, A., Raden, D. L., Bowman, D. S., Fay, F. S., and Davis, R. J. (1993) Serum-induced translocation of mitogen-activated protein kinase to the cell surface ruffling membrane and the nucleus. *J. Cell Biol.* **122,** 1089–1101.

3. Lenormand, P., Sardet, C., Pages, G., L'Allemain, G., Brunet, A., and Pouyssegur, J. (1993) Growth factors induce nuclear translocation of MAP kinases (p42^{mapk} and p44^{mapk}) but not their activator MAP kinase kinase (p45^{mapkk}) in fibroblasts. *J. Cell Biol.* **122,** 1079–1088.

4. Cavigelli, M., Dolfi, F., Claret, F. X., and Karin, M. (1995) Induction of c-fos expression through JNK-mediated TCF/Elk-1 phosphorylation. *EMBO J.* **14,** 5957–5964.

5. Kawasaki, H., Moriguchi, T., Matsuda, S., et al. (1996) Ras-dependent and Ras-independent activation pathways for the stress-activated-protein-kinase cascade. *Eur. J. Biochem.* **241,** 315–321.

6. Cheng, H. L. and Feldman, E. L. (1998) Bidirectional regulation of p38 kinase and c-Jun N-terminal protein kinase by insulin-like growth factor-I. *J. Biol. Chem.* **273,** 14,560–14,565.

7. Fukuda, M., Gotoh, Y., and Nishida, E. (1997) Interaction of MAP kinase with MAP kinase kinase: its possible role in the control of nucleocytoplasmic transport of MAP kinase. *EMBO J.* **16,** 1901–1908.

8. Rubinfeld, H., Hanoch, T., and Seger, R. (1999) Identification of a cytoplasmic-retention sequence in ERK2. *J. Biol. Chem.* **274,** 30,349–30,352.

9. Tanoue, T., Adachi, M., Moriguchi, T., and Nishida, E. (2000) A conserved docking motif in MAP kinases common to substrates, activators and regulators. *Nature Cell Biol.* **2,** 110–116.

10. Fukuda, M., Gotoh, I., Gotoh, Y., and Nishida, E. (1996) Cytoplasmic localization of MAP kinase kinase directed by its N-terminal, leucine-rich short amino acid sequence, which acts as a nuclear export signal. *J. Biol. Chem.* **271,** 20,024–20,028.

11. Adachi, M., Fukuda, M., and Nishida, E. (1999) Two co-existing mechanisms for nuclear import of MAP kinase: passive diffusion of a monomer and active transport of a dimer. *EMBO J.* **18,** 5347–5358.

12. Khokhlatchev, A. V., Canagarajah, B., Wilsbacher, J., et al. (1998) Phosphorylation of the MAP kinase ERK2 promotes its homodimerization and nuclear translocation. *Cell* **93,** 605–615.

13. Lenormand, P., Brondello, J. M., Brunet, A., and Pouyssegur, J. (1998) Growth factor-induced p42/p44 MAPK nuclear translocation and retention requires both MAPK activation and neosynthesis of nuclear anchoring proteins. *J. Cell Biol.* **142,** 625–633.

14. Adachi, M., Fukuda, M., and Nishida, E. (2000) Nuclear export of MAP kinase (ERK) involves a MAP kinase kinase (MEK)-dependent active transport mechanism. *J. Cell Biol.* **148,** 849–856.

15. Gaits, F., Degols, G., Shiozaki, K., and Russel, P. (1998) Phosphorylation and association with the transcription factor Atf1 regulate localization of Spc1/Sty1 stress-activated kinase in fission yeast. *Genes Dev.* **12,** 1464–1473.

16. Ferrell, J. E., Jr. (1996) Tripping the switch fantastic: how a protein kinase cascade can convert graded inputs into switch-like outputs. *Trends Biochem. Sci.* **21,** 460–466.

17. Lorenzen, J. A., Baker, S. E., Denhez, F., Melnick, M. B., Brower, D. L., and Perkins, L. A. (2001) Nuclear import of activated D-ERK by DIM-7, an importin family member encoded by the gene *moleskin*. *Development* **128,** 1403–1414.

18. Kim-Kaneyama, J., Nose, K., and Shibanuma, M. (2000) Significance of nuclear relocalization of ERK1/2 in reactivation of *c-fos* transcription and DNA synthesis in senescent fibroblasts. *J. Biol. Chem.* **275,** 20,685–20,692.

19. Lim, I. K., Hong, K. W., Kwak, I. H., Yoon, G., and Park, S. C. (2000) Cytoplasmic retention of p-Erk1/2 and nuclear accumulation of actin proteins during cellular senescence in human diploid fibroblasts. *Mech. Ageing Dev.* **119,** 113–129.

20. Aplin, A. E., Stewart, S. A., Assoian, R. K., and Juliano, R. L. (2001) Integrin-mediated adhesion regulates ERK nuclear translocation and phosphorylation of Elk-1. *J. Cell Biol.* **153,** 273–281.

21. Fincham, V. J., James, M., Frame, M. C., and Winder, S. J. (2000) Active ERK/MAP kinase is targeted to newly forming cell-matrix adhesions by integrin engagement and v-Src. *EMBO J.* **19,** 2911–2923.

22. Pritchard, C. A., Samuels, M. L., Bosch, E., and McMahon, M. (1995) Conditionally oncogenic forms of the A-Raf and B-Raf protein kinases display different biological and biochemical properties in NIH 3T3 cells. *Mol. Cell. Biol.* **15,** 6430–6442.

Study of MAPK Signaling Using Knockout Mice

Gilles Pagès and Jacques Pouysségur

1. Introduction

The p42 and p44 mitogen-activated protein kinases (MAPKs) (p42/p44 MAPK, Erk2 /Erk1) are activated through the small G protein Ras and sequential activation of the protein kinases Raf and MEK on stimulation of cells with a broad range of extracellular signals *(1,2)*. This signaling pathway, conserved in evolution, controls cell fate, differentiation, proliferation, and cell survival in various invertebrates, mammalian cells, and plants *(3–7)*. To understand the specific role of the commonly expressed and activated p42 and p44 MAPK isoforms in the whole animal, we generated p44 MAPK–deficient mice through homologous recombination in embryonic stem (ES) cells *(8)*. p44 MAPK null mice are viable, fertile, and of normal size. This result indicates that p44 MAPK is dispensable and that the second isoform, p42 MAPK, can compensate for the loss of p44 MAPK. Loss of the p44 MAPK isoform does not affect expression of p42 MAPK in all the tissues tested even in the sciatic nerve, the only tissue in which p44 MAPK is expressed at higher levels than p42 MAPK *(1)*. We previously established that p42/p44 MAPK nuclear translocation *(9)* and persistent activation during the G1 phase of the cell cycle is a prerequisite for growth control in fibroblasts *(10)*. Thus, it was crucial to analyze the temporal activation of p42/p44 MAPKs and reinitiation of DNA synthesis in wild-type and p44 MAPK–deficient mouse embryo fibroblasts (MEFs). The growth rate as well as reinitiation of DNA synthesis in serum-starved MEFs is not significantly impaired by the ablation of both alleles of p44 MAPK gene *(8)*. Although the amount of p42 MAPK is unchanged in p44 MAPK$^{-/-}$ MEFs, examination of the time course of p42 MAPK activation, in particular in mid- (4 h) or late- (8 h) G1 phase, reveals more sustained activation than in wild-type MEFs *(8)*. Total MAPK activation (Erk1 + Erk2) is identical in wild-type and deficient MEFs after long-term activation. This result indicates that the intensity of the

signal matters more than the quality of the MAPK isoform initiating the long-term signaling, and that, for cell-cycle progression, p42 MAPK can fully substitute for the lack of p44 MAPK. However, a closer analysis of p44 MAPK$^{-/-}$ mice shows that thymocyte maturation beyond the CD4$^+$/CD8$^+$ stage is reduced by half, with a similar diminution in the CD3^{high} thymocyte subpopulation **(8)**. We also observed a severe reduction in thymocyte proliferation following activation with an anti-TcR monoclonal antibody (MAb) in the presence of phorbol myristate acetate (PMA) even though p42 MAPK activation is more sustained in these cells. Our result demonstrates that the MAPK signal threshold value required for thymocyte maturation and proliferation is not achieved in the absence of p44 MAPK, reflecting a role of this kinase in thymocyte development.

An identical approach developed for the deletion of the second isoform, p42 MAPK, led to a more drastic result. Whereas the heterozygotes were viable and fertile, the null p42 MAPK mice died during development. This result already indicates that p44 MAPK cannot substitute for the lack of p42 MAPK, suggesting either that p42 MAPK has a set of specific substrates, or that the levels of p44 MAPK are too low to compensate. The second hypothesis, currently tested, implies that crossing transgenic mice overexpressing p44 MAPK should rescue the p42 MAPK null mice.

2. Materials

2.1. Preparation of Gelatin

Add 0.1 g of gelatin/100 mL of H$_2$O. Autoclave to sterilize and store in aliquots of 100–250 mL at 4°C.

2.2. Production of Leukemia Inhibitory Factor

Leukemia inhibitory factor (LIF) was produced by transfecting Cos-7 cells with a plasmid coding for LIF. The activity of LIF in the conditioned medium is measured by its ability to inhibit ES cell differentiation. One unit of LIF is the dilution that gives detectable inhibition of differentiation. LIF activity in the transfected Cos cell supernatant should be 5–20 × 10^4 U/mL. LIF is stored at –20°C in 0.5- and 1-mL aliquots.

2.3. Propagation of ES Cells

The ES cells (E14Tg2a.IV ES cells) used in our experiments were initially provided by Dr. Martin Hooper (Edinburgh, UK) and subcloned in Dr. Austin Smith's laboratory (Edinburgh, UK). Add 5 mL of gelatin solution per 25-cm^2 tissue culture flask and incubate for at least 1 h at 37°C. Seed 10^6 ES cells into a 25-cm^2 gelatin-coated flask in 10 mL of prewarmed Dulbecco's modified

Eagle's medium (DMEM) supplemented with 2-mercaptoethanol (10^{-4} *M*), 10% fetal bovine serum (FBS), and 100 U/mL of LIF. Change the medium every 24 h and add fresh LIF at each change.

2.4. Recombination Vector

The p44 MAPK gene was obtained by screening a genomic library of SV129 mice *(11)*. This was essential for a high efficiency of homologous recombination in ES cells derived from SV 129 mice. The gene spans approx 8 kb and can be divided into nine exons and eight introns, each coding exon contains from one to three of the highly conserved protein kinase domains. We chose to replace a region comprising and area between a *Sac*I site present in the second intron and an *Afl*II site present in the extreme part of the third exon by the PGK NEO cassette (**Fig. 1**). The third exon contains the region coding for the threonine and tyrosine residues phosphorylated on MAPK activation *(1,10,12)*. We have also added at both extremities the HSV Thymidine Kinase gene (HSV TK). PGK NEO in the presence of G418 allows for positive selection and the HSV TK gene allows for negative selection in the presence of Gancyclovir. The HSV TK gene metabolizes Gancyclovir into a toxic compound that kill the cells. Mansour et al. *(13)* developed this strategy to counterselect ES clones in which the targeting vector has been randomly integrated into genomic DNA. On homologous integration, the HSV TK gene is lost and cells can survive in the presence of Gancyclovir.

2.5. DNA Preparation From Culture Cells or Mice Tail Clippings

Wash the wells for genotype analysis twice in ice-cold phosphate-buffered saline (PBS) and lyse the cells in 100 mL of lysis solution (100 m*M* Tris-HCl, pH 7.5; 10 m*M* EDTA; 100 m*M* NaCl; 0.1% sodium dodecyl sulfate [SDS]; and 0.1 mg/mL of proteinase K). After 16 h at 56°C, precipitate the DNA by 1 vol of isopropanol and resuspend in 100 μL of H_2O. Apply the same procedure to 1-cm mice tail clippings. In that case, use 500 μL of lysis buffer and resuspend the DNA in 300 μL of H_2O.

2.6. Other Reagents and Equipment

1. LIF (*see* **Subheading 2.2.**), appropriate restriction enzymes, blotting equipment, and antibodies.
2. DMEM containing 2-mercaptoethanol (10^{-4} *M*) and 10% FBS.
3. Six-well dish.
4. DMEM/Ham's F12, 1:1 and [^{3}H] thymidine.
5. Protein determination BCA kit (Pierce), anti-CD4-PE, anti-CD8-FITC, and Cy-Chrome-antimouse αβTCR-Cy MAbs for fluorescent-activated cell sorting.
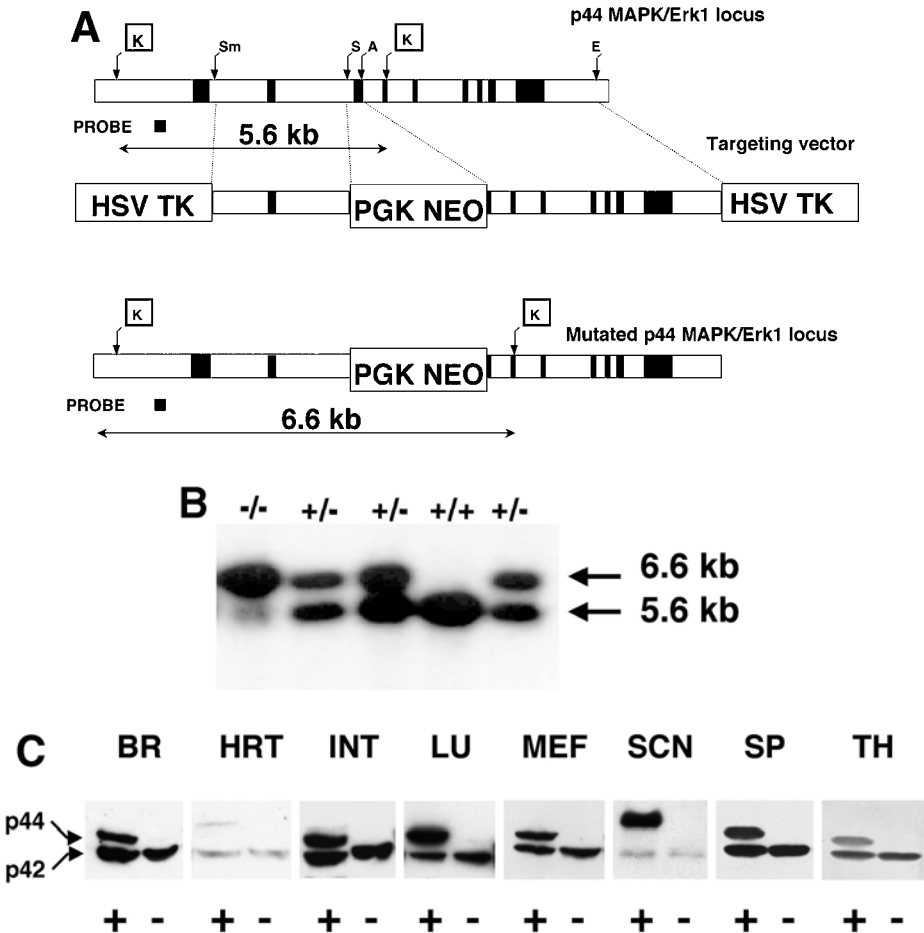
Fig. 1. Generation of p44 MAPK (Erk1)-deficient mice. **(A)** Schematic representation of the genomic p44 MAPK locus. The probe used for Southern analysis and the restriction enzyme sites are shown (K, *Kpn*I; S, *Sac*I; A, *Afl*II; E, *Eco*RI; Sm, *Sma*I). **(B)** Southern blot analysis of genomic DNA from five littermates issued from p44 MAPK heterozygote crosses. Wild-type (5.6 kb) and disrupted (6.6 kb) *Kpn*I fragments are visualized together with genotypes. **(C)** MAPK immunoblot analysis confirmed the absence of the p44 MAPK protein. Extracts of brain (BR), heart (HRT), intestine (INT), lung (LU), embryo fibroblasts (MEF), sciatic nerve (SCN), spleen (SP), and thymus (TH) were examined by immunoblot analysis with a polyclonal antibody directed against both p42/p44 MAPKs *(14)*.

3. Methods

3.1. Manipulation of ES Cells

ES cells in which the gene of interest should be inserted (*see* **Note 1**) are derived directly from the pluripotent inner cell mass of the preimplantation mouse embryo. They are permanent cell lines that can be propagated and experimentally manipulated in vitro. The unique feature of ES cells is that they retain the properties of normal early embryo cells and thus can be reintroduced into the blastocyst, where they participitate fully in embryonic development. ES cells contribute to differentiated progeny of tissues, including the production of functional gametes. The latter permits germline transmission of the ES cell genotype and thereby provides a system for introducing homologous recombination events into experimental animals. To maintain ES cells in a pluripotent state during in vitro manipulation, they must be cultured in the presence of LIF.

3.2. Transfection of ES Cells and Verification of Targeted Disruption of the p44 MAPK Gene

1. Wash trypsinized ES cells in culture medium and suspend in PBS.
2. Place the cell suspension in an electroporation cuvet (Bio-Rad, Hercules, CA), mixed with homologous recombination vector linearized by *Xmn*I at a concentration of 25 µg/mL, electroporate at 500 V, and 40 µF, and plate on dishes.
3. After 2 d, add G418 at 400 µg/mL and Gancyclovir at 2×10^{-6} *M (13)* to the culture medium and pick the surviving colonies after another 15 d.
4. Expand each clone in duplicate 24-well dishes. Digest approx 5 µg of DNA with *Kpn*I (one site in the promoter and one site in the fourth exon), and submit to Southern blot analysis. The probe corresponds to a 700-bp *Pst*I/*Pst*I fragment of the promoter region. The wild-type allele generates a 5.6-kb fragment and the mutated allele a 6.6-kb fragment because of the PGK NEO cassette insertion (**Fig. 1B**).
5. *Optional:* The ES cell genotype can be examined by polymerase chain reaction (PCR) with the following oligonucleotides: primer 1 located in the neomycin gene: 5' GAA GGA GCC AAG CTG CTA TT; primer 2 located in exon 4: 5' AAC GTG TGG CTA CGT ACT C; and primer 3 located in exon 3: 5' AGC AAT GAC CAC ATC TGC TA. The wild-type fragment is 330 bp and the targeted fragment is 600 bp (data not shown). All three primers are added to the PCR reaction at the same time.
6. Add 200 ng of genomic DNA (vortexed well to induce some shearing) to a 50-µL PCR reaction.

7. Add 1 U of *Taq* polymerase and 0.2 m*M* of dNTPs. The conditions are 96°C for 25 s, 56°C for 25 s, and 72°C for 60 s for 30 cycles. The same protocol was used for genotyping the mice except that DNA was prepared from a 1-cm tail piece.

8. *Optional:* Genotyping of homozygous mice can also be performed by Western blot analysis. Homogenize a 1-cm tail piece in Laemmli sample buffer. Resolve approx 40 μg of protein on a 10% SDS-polyacrylamide gel electrophoresis (PAGE) and submit to Western blotting with an anti p42/p44 MAPK antibody (Zymed).

3.3. Blastocyst Injection and Animal Breeding

1. Recover blastocyts from C57 BL6 mice.
2. Inject blastocysts with ES cells and transfer into the uterus of pseudopregnant F1 (C57 BL6) mice.
3. Breed male chimeras with 129/Ola or MF1 females to test for germline transmission.
4. Intercross F1 (129/Ola x 129 Ola or MF1 x 129/Ola) mice heterozygous for the mutation to produce homozygotes.
5. Cage mice in rooms with a 12-h light and 12-h darkness cycle. Allow free access to water and give a standard mouse diet (*see* also **Note 2**).

3.4. Generation of MEFs

1. Extract from yolk sac 14-d wild-type, heterozygous, and homozygous embryos obtained by intercrossing heterozygous mice.
2. Remove the head and heart and cut the remaining embryo into small pieces with a sterile scalpel.
3. Aspirate "fragments" through a 1-mL pipet tip in DMEM containing 2-mercaptoethanol (10^{-4} *M*) and 10% FBS.
4. Plate the grounded tissues on 6-cm dishes. At this step, cells should grow rapidly and three to five passages can be performed. Cell density is crucial at this step because cells cannot grow efficiently at low density.
5. Trypsinize confluent cells (approx 5×10^6 cells in 100-mm plates) with trypsin/EDTA, and dilute in three new gelatinized plates. After passage six, cells undergo a "pseudocrisis" and their growth rate decreases. After this period, many immortalized cells can grow. Many MEFs derived from one initial embryo before "crisis" or after immortalization were tested for their growth capacity and for their ability to activate p42 and p44 MAPK in response to growth factors (for more details and explanations, *see* **Note 3**).

3.5. Determination of Growth Capacity of Cells Derived From Wild-Type or p44 MAPK-Deficient Mice

3.5.1. MEF

3.5.1.1. Growth Curves

1. Plate freshly isolated MEFs (second passage) at a density of 10^5 cells per six-well dishes.
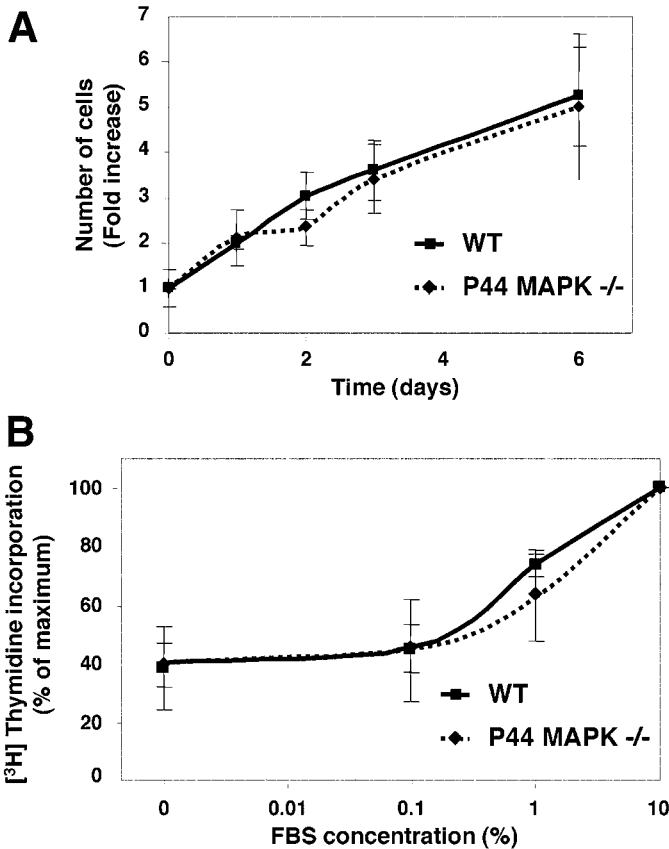
Fig. 2. Analysis of growth capacity of wild-type or p44 MAPK–deficient MEFs. **(A)** MEFs of early passages (three to five) obtained from six wild-type and four p44 MAPK-deficient embryos were plated in duplicate at a density of 10^5 cells/six-well plates. One, 2, 3, and 6 d after plating, cells were trypsinized and counted in a Coulter counter (Coultronics). We plotted the increment in cell number for each day. **(B)** Confluent MEFs of early passages (three to five) obtained from eight wild-type and six p44 MAPK-deficient embryos in 24-well plates were serum deprived for 48 h and then stimulated with different concentrations of FBS (triplicate for each serum concentration). Radioactivity (counts per minute) incorporated into DNA in the presence of 10% FBS was considered as 100%. The results are expressed as a percentage of the maximal [^{3}H]-thymidine incorporation.

2. Count the cells 16 h later and take this number of cells as the d 1 reference.
3. Trypsinize and count the cells every 24 h during 7 d cells. Determine the increment in cell number and plot against the reference time (**Fig. 2A**).

3.5.1.2. Reinitiation of DNA Synthesis (*see also* **Note 4**)

1. Plate freshly isolated MEFs (second passage) on 24-well plates and grow to confluency.
2. Serum deprive the MEFs for 48 h in serum-free medium (DMEM/Ham's F12 [1:1]) to undergo quiescence.
3. Stimulate with increasing serum concentrations in DMEM/Ham's F12 containing 0.5 μCi/mL of [^{3}H]-thymidine.
4. After a 24-h incubation, fix and wash the cells four times with cold 5% trichloroacetic (TCA) acid.
5. Measure the radioactivity incorporated into TCA-precipitable material after solubilization in 0.1 *M* NaOH (**Fig. 2B**).

3.5.2. Thymocytes

1. Dissect the thymus of control or p44 MAPK-deficient mice and homogenize with a syringe pestle on a piece of gauze adapted on a 50-mL Falcon tube.
2. Obtain cells by flowing RPMI medium supplemented with 10% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin through the gauze and count the cells.
3. Plate thymocytes (2×10^5) in 200 μL of RPMI medium supplemented with 10% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin.
4. Stimulate the cells with the following concentration of mitogens: 10 ng/mL of PMA or rat antimouse CD3e (precoated at 10 mg/mL) or both for 72 h. Add 1 μCi of [^{3}H]-thymidine for the last 18 h and then measure its incorporation into DNA (**Fig. 3**).

3.6. Preparation of Protein Extracts

3.6.1. Tissues

1. Dissect different tissues from wild-type and p44 MAPK-deficient mice and immediately freeze in liquid nitrogen.
2. Homogenize each tissue using an Ultra-Turrax (Fisher Bioblock Scientific) in lysis buffer.
3. Eliminate debris by centrifuging at 12,000*g* for 30 min at 4°C and at 100,000*g* for 20 min at 4°C.
4. Estimate protein concentrations with the BCA kit (Pierce), and store the extracts at –80°C.

3.6.2. Cells

1. Wash MEFs or thymocytes twice with ice-cold PBS and lyse in the same buffer given above.
2. Estimate the protein concentration with the BCA kit.

3.7. Western Blot

1. Resolve proteins from tissues or cell lysates on SDS polyacrylamide gels (10 %) and electrophoretically transfer to immobilon membranes.
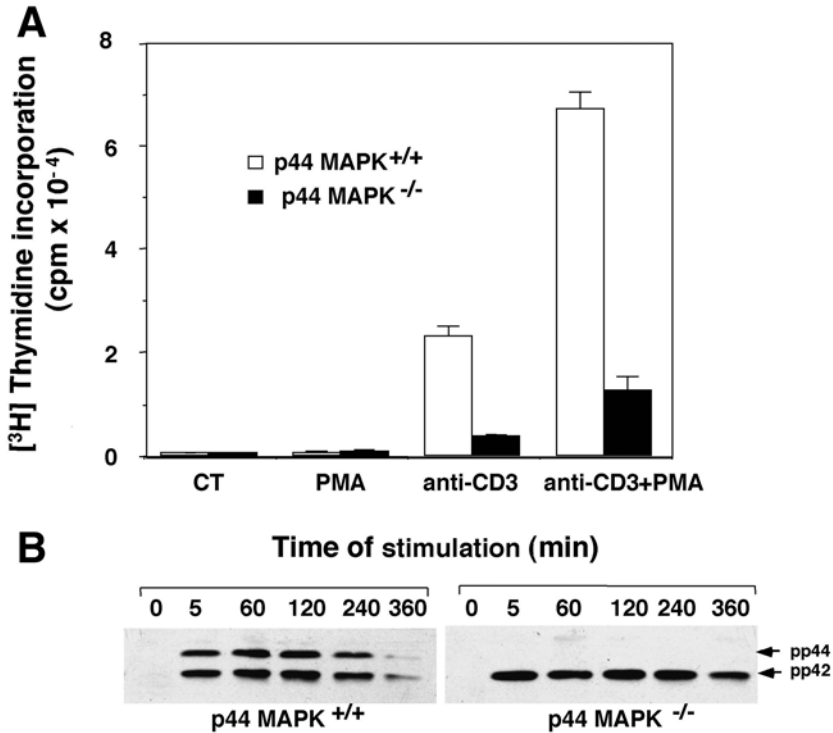
Fig. 3. Defect in proliferation of p44 MAPK$^{-/-}$ thymocytes. **(A)** Total thymocytes from control or p44 MAPK-deficient mice were isolated and stimulated with crosslinked anti-CD3ε MAb alone or in combination with PMA in 96-well microplaques. Thymocytes (2×10^5) were plated in 200 μL of medium. Cells were stimulated with the indicated concentrations of mitogens: 10 ng/mL of PMA and/or with rat antimouse CD3 (plated at 10 μg/mL) for 72 h. [^{3}H]-Thymidine (1 μCi) was added for the last 18 h. [^{3}H]-Thymidine incorporation was determined. Results are the mean +/– SEM of three different experiments performed in quadruplicate. **(B)** Activation of p42/p44 MAPKs in wild-type and p44 MAPK-deficient thymocytes. Thymocytes were stimulated with anti-CD3ε MAb + PMA for the indicated times. Proteins from cell lysates were separated by SDS-PAGE and transferred to Immobilon membranes for Western blotting with antiphosphospecific MAPK antibodies. Immunoreactivity was detected by enhanced chemiluminescence.

2. For immunoblotting, use either a polyclonal antibody or MAb directed against the phosphorylated forms of p42/p44 MAPK or a polyclonal antibody to the 15 last amino acids of p44 MAPK that recognizes both p42 and p44 MAPK (*see* **Figs. 1C**, **3B**, and **4**) *(14)*.
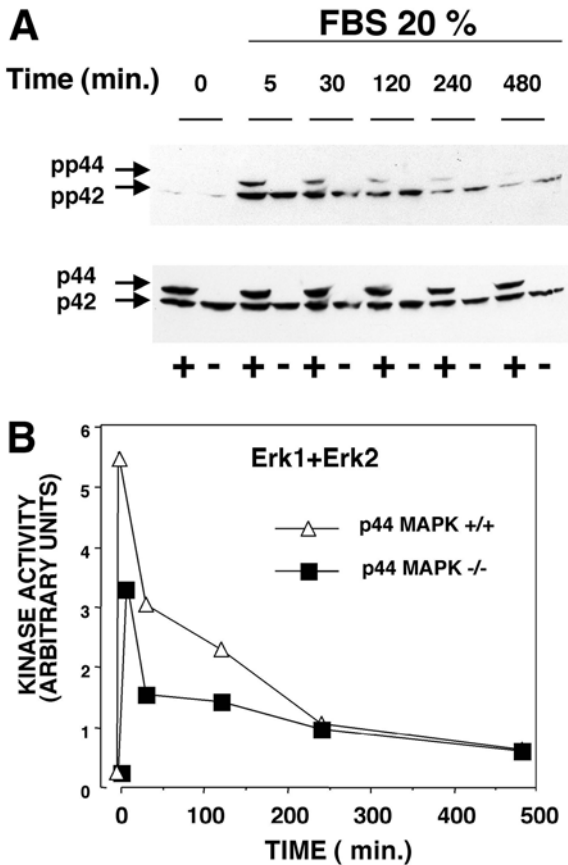
Fig. 4. Time course of serum-stimulated MAPK activity in wild type and p44 MAPK-deficient MEFs. **(A)** MEFs from early passages (three to five) were serum deprived for 24 h and subsequently stimulated with 20% FBS for the different indicated times. Total cell extracts were separated by SDS-PAGE (7.5% gels) and examined by immunoblot using either a MAb directed against phospho-MAPKs (top) or a polyclonal antibody that recognizes the C-terminal domain of both MAPK isoforms (bottom) *(14)*. **(B)** Quantification of phosphorylated p42 and p44 MAPK in wild-type and p44 MAPK in deficient MEFs.

3.8. Flow Cytometry Analysis

1. Stain thymocytes from 6-wk-old mice with saturating concentrations of anti-CD4-PE, anti-CD8-FITC, and Cy-Chrome-antimouse αβTCR-Cy MAbs at 4°C for 30 min in 100 μL of PBS containing 0.1% bovine serum albumin.
2. Perform analysis using a FACscan flow cytometer (Becton-Dickinson).

4. Notes

1. One prerequisite to homologous recombination experiments is cloning the gene of interest. To increase the percentage of homologous recombinants, it is absolutely necessary to clone the gene of interest from a genomic library constructed with isogenic DNA isolated from the ES cells that will serve for gene targeting *(16)*. This genomic sequence is used to prepare the targeting vector. In Southern analysis, we sometimes observed parasite fragments detected by probes corresponding to the coding region. This problem is owing to the fact that p44 MAPK probes can detect restriction fragments of the corresponding p42MAPK gene as well as fragments corresponding to a pseudo p44 MAPK gene that is intron-less (Gilles Pagès, personal communication). Finally, the best probe for genotype analysis corresponds to a fragment of the p44 MAPK gene promoter *(11)*. The presence of this intron-less gene has also caused numerous problems for genotyping by PCR. We finally have access to the mouse genome sequencing program and have designed specific oligonucleotides described in **Subheadings 2.** and **3.** (Gilles Pagès and Jeff Molkentin, personal communication).

2. For all experiments, the use of commercially obtained animals is not considered to be good control. It is better to bread heterozygous animals as controls and to use wild-type or heterozygous of the same litter.

3. MEFs were isolated from different embryos to obtain statistically significant analysis of the effects of the mutation. Furthermore, to avoid clonal particularities that could occur after the "pseudocrisis," it is particularly important to analyze the different MEFs before the crisis that generally happens after passage five to six. For reinitiation of DNA synthesis, cells must be serum deprived for at least 2 d in order to lower the basal thymidine incorporation, which is generally high in these cells. We also removed potential secreted growth factors by rinsing the cells two times with serum-free medium 1 d after the first serum removal. By doing this, basal thymidine incorporation is lowered even further. For growth curves, because of their low plating efficiency, MEFs must be seeded at a density no lower than 10^4 cells/cm^2.

4. We sometimes observed modifications in the basal level of [^{3}H]-thymidine incorporation. This was probably dependent on the following:
   a. Animal house facilities: The problem of a high basal level occurred at the same time as bacterial infection.
   b. Excessive pipeting during cell plating: it is very important not to pipet the cells up and down numerous times. The shear stress engendered by pipetting the cells activates many signaling pathways, in particular the p42/p44 MAPK pathway. We prefer to prepare a cell solution at the appropriate density in a 50-mL Falcon tube and to gently agitate the tube to maintain a homogeneous cell solution before plating.

References

1. Boulton, T. G., Nye, S. H., Robbins, D. J., et al. (1991) ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell* **65,** 663–675.

 2. Ahn, N. G., Seger, R., and Krebs, E. G. (1992) The mitogen-activated protein kinase activator. *Curr. Opin. Cell Biol*. **4,** 992–999.
 3. Nishida, E., and Gotoh, Y. (1993) The MAP kinase cascade is essential for diverse signal transduction pathways. *Trends Biochem. Sci*. **18,** 128–131.
 4. Marshall, C. J. (1995) Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80,** 179–185.
 5. Brunet, A. and Pouyssegur, J. (1997) Mammalian MAP kinase modules: how to transduce specific signals. *Essays Biochem*. **32,** 1–16.
 6. Ligterink, W. and Hirt, H. (2001) Mitogen-activated protein (MAP) kinase pathways in plants: versatile signaling tools. *Int. Rev. Cytol*. **201,** 209–275.
 7. Bent, A. F. (2001) Plant mitogen-activated protein kinase cascades: negative regulatory roles turn out positive. *Proc. Natl. Acad. Sci. USA* **98,** 784–786.
 8. Pagès, G., Guerin, S., Grall, D., et al. (1999) Defective thymocyte maturation in p44 MAP kinase (Erk 1) knockout mice. *Science* **286,** 1374–1377.
 9. Brunet, A., Roux, D., Lenormand, P., et al. (1999) Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. *EMBO J*. **18,** 664–674.
10. Pagès, G., Lenormand, P., L'Allemain, G., et al. (1993) Mitogen-activated protein kinases p42*mapk* and p44*mapk* are required for fibroblast proliferation. *Proc. Natl. Acad. Sci. USA* **90,** 8319–8323.
11. Pagès, G., Stanley, R., Legal, M., et al. (1995) The mouse p44 Mitogen-Activated Protein kinase (extracellular signal-regulated kinase 1) gene. *J. Biol. Chem.* **270,** 26,986–26,992.
12. Boulton, T. G., Yancopoulos, G. D., Gregory, J. S., et al. (1990) An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. *Science* **249,** 64–67.
13. Mansour, S. L., Thomas, K. R., and Capecchi, M. R. (1988) Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. *Nature* **336,** 348–352.
14. McKenzie, F. R. and Pouyssegur, J. (1996) cAMP-mediated growth inhibition in fibroblasts is not mediated via mitogen-activated protein (MAP) kinase (ERK) inhibition. cAMP-dependent protein kinase induces a temporal shift in growth factor-stimulated MAP kinases. *J. Biol. Chem*. **271,** 13,476–13,483.
15. Guerin, S., Mari, B., Fernandez, E., et al. (1997) CD10 is expressed on human thymic epithelial cell lines and modulates thymopentin-induced cell proliferation. *FASEB J*. **11,** 1003–1011.
16. te Riele, H., Maandag, E. R., and Berns, A. (1992) Highly efficient gene targeting in embryonic stem cells through homologous recombination with isogenic DNA constructs. *Proc. Natl. Acad. Sci. USA* **89,** 5128–5132.

12

Computer Simulation of MAPK Signal Transduction

Baltazar D. Aguda and Herbert M. Sauro

1. Introduction

We predict that computational modeling platforms will soon become standard tools in experimental laboratories involved in the study of complex regulatory networks of various cellular processes—a field of research where an avalanche of genomic, proteomic and other biochemical data have recently been gleaned, and this trend is expected to continue in the foreseeable future. Quantitative kinetic modeling requires one to postulate detailed molecular pathways and to carry out analyses in the context of an integrated dynamic system so that predictions can be made and compared with experimental data as well as aid in the design of future experiments. The speed of modern computers is such that one can perform simulations of many possible models and discriminate against those that are less probable.

The primary objective of this chapter is to guide the newcomer through the basic steps in computational modeling of biochemical reaction networks, starting from a molecular mechanism that is amenable to quantitative description and moving on to coding the kinetic equations in a computer file so that a computer program can then solve the associated dynamical equations. Readers who are already familiar with computer simulations will also benefit from reading our notes on the essential features that we think, based on experimental evidence, must be observed in model mechanisms of mitogen-activated protein kinase (MAPK) signaling. We use the model of Huang and Ferrell *(1)* to illustrate the detailed procedure in our computer simulation of the dynamics and steady-state behavior of MAPK signaling. Our purpose here is not to validate the Huang-Ferrell model but to present a method of computer simulation that could be useful to the experimentalist. Other models are also noted, and it is hoped that the reader can analyze or modify them using the methods learned in this chapter. We also include a short list of computer software available on

the internet and deemed to be user-friendly, especially for biologists who do not have the time to learn the intricacies of computer programming.

2. Materials

The kinetic model we solve in the next section requires a computer program that integrates a system of ordinary differential equations. Because of its speed, ease of use, and many convenient features, we have chosen the software called Berkeley Madonna™ (BM) developed by Robert Macey and George Oster of the University of California at Berkeley. Information on purchasing and downloading this inexpensive program from the internet can be found at www.berkeleymadonna.com. BM can be run in either a Windows or Macintosh environment. BM for Windows requires a personal computer with at least an Intel x86 or compatible processors that operate using Microsoft Windows 95, Windows NT 4.0, or later versions. BM for Macintosh runs on Apple Power Macintosh and 100% compatible computers with a PowerPC CPU. Note that other recommended software platforms are given in **Table 1** and can be used instead of BM (*see* **Note 1**).

3. Methods

3.1. Creating the Equations File

We use the model mechanism of Huang and Ferrell *(1)* to illustrate the detailed procedure of coding the kinetic equations into BM. The reaction network diagram and a brief summary of the model are given in **Fig. 1** (*see* **Note 2**). When BM is run for the first time, an equation window is opened wherein the user types the kinetic equations and other computational details of the model. For the Huang–Ferrell mechanism, the corresponding file is given in **Table 2**. Because it is assumed that the total protein concentrations of each level of the cascade are constant (denoted by the parameters *tot1*, *tot2*, and *tot3* in **Table 2**), we classify protein concentrations as either independent or dependent dynamic variables in our kinetic equations. For example, concentration of MKKK (denoted as *mkkk* in **Table 2**) is considered dependent because it could be derived from *tot3* and the concentration of MKKK* (denoted as *mkkks*). The concentrations of MKK-P, MKK-PP, MAPK-P, and MAPK-PP are the other independent variables (whose concentration symbols in **Table 2** are *mkkp*, *mkkpp*, *mapkp*, and *mapkpp*, respectively), and the other dependent species and their corresponding concentrations (in parentheses) are MKK (*mkk*) and MAPK (*mapk*). The individual reaction steps are endowed with the rate expressions identified as *v1*, *vm1*, *v2*, *vm2*, *v3*, *vm3*, *v4*, *v4*, *v5*, and *vm5* in **Table 2** (*see* **Note 3**). These rate expressions are assumed to be of the Michaelis-Menten type; for example, *v1 = k1*e1*mkkk/(j1 + mkkk)* in which *j1* is the

Table 1
Computer Simulation Software for Biochemical Reaction Networks

Software	Web site
Berkeley Madonna	www.berkeleymadonna.com/
MCell	www.mcell.cnl.salk.edu/
E-Cell	http://e-cell.org
Virtual Cell	www.nrcam.uchc.edu/index.html
Gepasi	www.gepasi.org/gepasi.html
SCAMP	www.sys-bio.org
Jarnac	www.sys-bio.org
DBsolve	http://websites.ntl.com/~igor.goryanin/
Cellerator	*see* **ref. 7**

Michaelis constant, $e1$ is the total concentration of the enzyme catalyzing reaction 1, and $k1$ is a parameter.

The equations file (**Table 2**) has five differential equations (indicated by d/dt, which signifies rate of change with time), the equations of the dependent variables in terms of independent variables, the rate expressions (the vs), the initial values of the independent variables (indicated by "*init*"), and the values of the parameters of the model. In addition, the first few lines of the equations file contain the statements for the integration METHOD used (in this case the integrator for "STIFF" systems, which have widely varying values of parameters; other integrators are available in BM), the initial value of time (STARTTIME), the final value of integration time (STOPTIME), and the time step for integration (DT).

3.2. Running the BM Program

BM provides a good tutorial and user guide so we only give a very brief sample of the software's capabilities here. To run the program, simply click on the "Run" button on the equations window; the program will then open a window that gives a graph of concentration versus time for selected species (e.g., **Fig. 2** shows *mapkpp* vs *time*). One feature of BM that is quite convenient for exploring the effect of changing parameters is the parameter slider. Click on the "Parameters" menu and select "Define Sliders," which opens a window where you could select which parameters to vary. **Figure 2** shows the sliders for $e1$ and STOPTIME. It is convenient to choose STOPTIME as a slider when one is interested in following the change in concentrations up to their steady-state levels, which is what we do next.

Table 2
The Equations File for the Huang–Ferrell Model Used by Berkeley Madonna

METHOD STIFF
STARTTIME = 0
STOPTIME = 100
DT = 0.01

$d/dt(mkkks) = v1 - vm1$
$d/dt(mkkp) = v2 + vm3 - (vm2 + v3)$
$d/dt(mkkpp) = v3 - vm3$
$d/dt(mapkp) = v4 + vm5 - (vm4 + v5)$
$d/dt(mapkpp) = v5 - vm5$

$mkkk = tot3 - mkkks$
$mkk = tot2 - (mkkp + mkkpp)$
$mapk = tot1 - (mapkp + mapkpp)$

$v1 = k1*e1*mkkk/(j1 + mkkk)$
$vm1 = km1*e2*mkkks/(jm1 + mkkks)$
$v2 = k2*mkkks*mkk/(j2 + mkk)$
$vm2 = km2*kkpase*mkkp/(jm2 + mkkp)$
$v3 = k3*mkkks*mkkp/(j3 + mkkp)$
$vm3 = km3*kkpase*mkkpp/(jm3 + mkkpp)$
$v4 = k4*mkkpp*mapk/(j4 + mapk)$
$vm4 = km4*kpase*mapkp/(jm4 + mapkp)$
$v5 = k5*mkkpp*mapkp/(j5 + mapkp)$
$vm5 = km5*kpase*mapkpp/(jm5 + mapkpp)$

$init\ mkkks = 0.0$
$init\ mkkp = 0.0$
$init\ mkkpp = 0.0$
$init\ mapkp = 0.0$
$init\ mapkpp = 0.0$

$tot3 = 3.0$
$tot2 = 1200.0$
$tot1 = 1200.0$
$e1 = 0.1$
$e2 = 0.3$
$kkpase = 0.3$
$kpase = 120.0$
$j1 = 300.0$
$jm1 = 300.0$

Table 2 *(continued)*

j2 = 300.0
jm2 = 300.0
j3 = 300.0
jm3 = 300.0
j4 = 300.0
jm4 = 300.0
j5 = 300.0
jm5 = 300.0
k1 = 1.0
km1 = 1.0
k2 = 1.0
km2 = 1.0
k3 = 1.0
km3 = 1.0
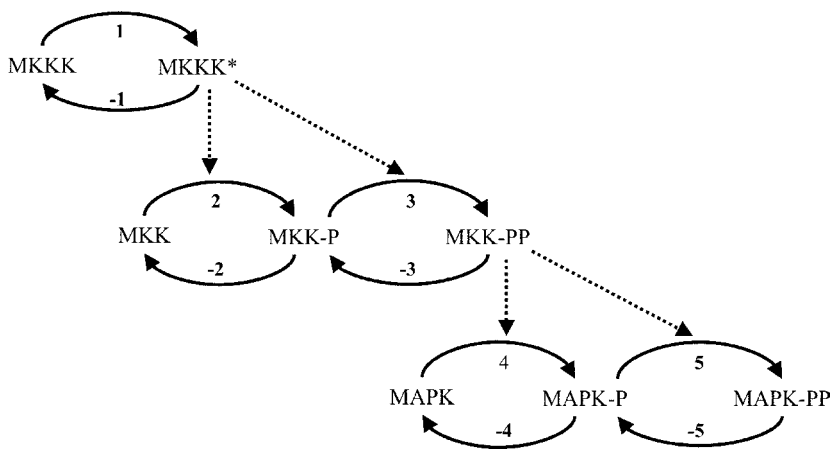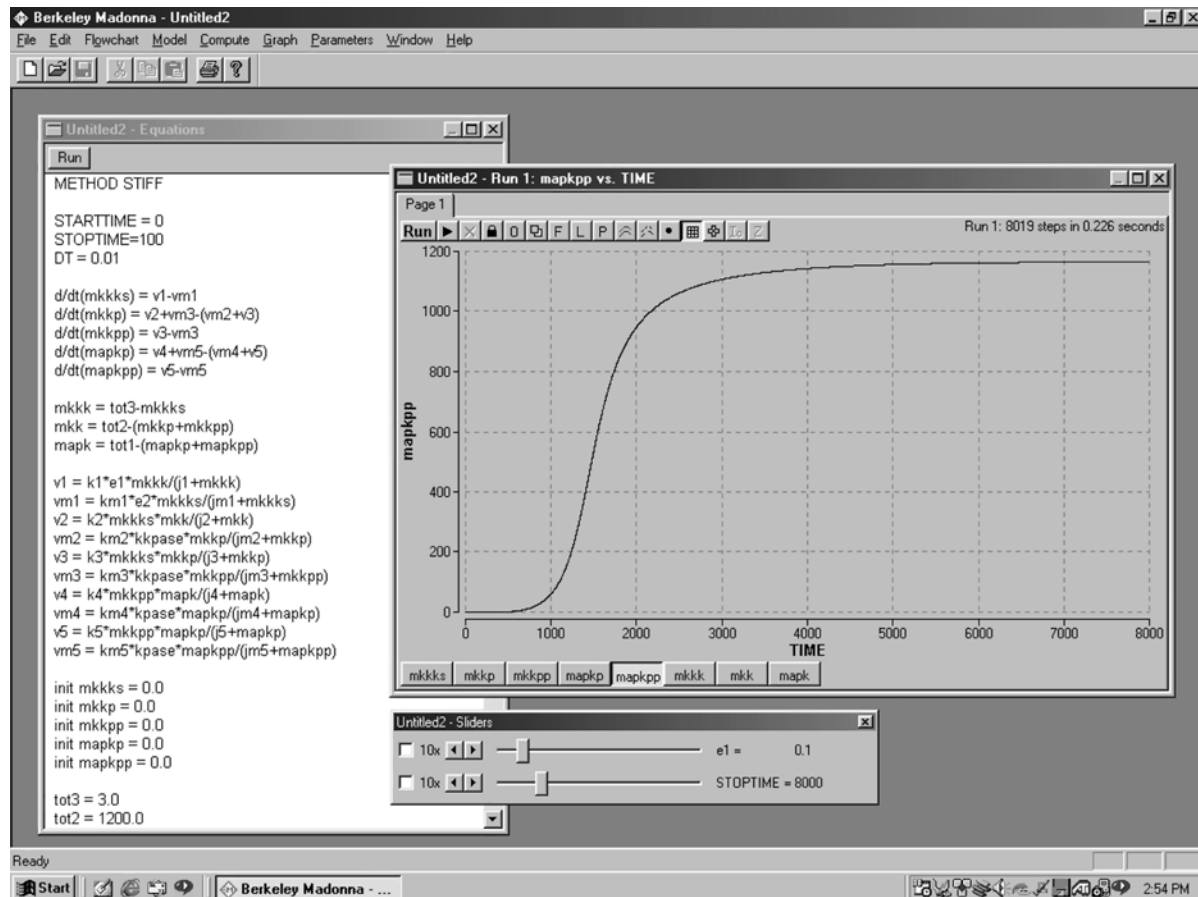*k4* = 1.0
km4 = 1.0
k5 = 1.0
km5 = 1.0

Fig. 1. Huang–Ferrell model for the MAPK cascade. Step 1, the activation of MKKK, is catalyzed by an enzyme E1; the reverse, step –1, is catalyzed by an enzyme E2. Steps 2 and 3 represent the two-collision, nonprocessive mechanism for the double phosphorylation of MKK; these steps are catalyzed by MKKK* while their reverse are assumed to be catalyzed by a phosphatase KKPase. Likewise, steps 4 and 5 represent the double phosphorylation of MAPK catalyzed by the active MKK-PP, and the reverse steps are catalyzed by a phosphatase KPase.

Berkeley Madonna - Untitled2

File Edit Flowchart Model Compute Graph Parameters Window Help

Untitled2 - Equations

Run

```
METHOD STIFF

STARTTIME = 0
STOPTIME=100
DT = 0.01

d/dt(mkkks) = v1-vm1
d/dt(mkkp) = v2+vm3-(vm2+v3)
d/dt(mkkpp) = v3-vm3
d/dt(mapkp) = v4+vm5-(vm4+v5)
d/dt(mapkpp) = v5-vm5

mkkk = tot3-mkkks
mkk = tot2-(mkkp+mkkpp)
mapk = tot1-(mapkp+mapkpp)

v1 = k1*e1*mkkk/(j1+mkkk)
vm1 = km1*e2*mkkks/(jm1+mkkks)
v2 = k2*mkkks*mkk/(j2+mkk)
vm2 = km2*kkpase*mkkp/(jm2+mkkp)
v3 = k3*mkkks*mkkp/(j3+mkkp)
vm3 = km3*kkpase*mkkpp/(jm3+mkkpp)
v4 = k4*mkkpp*mapk/(j4+mapk)
vm4 = km4*kpase*mapkp/(jm4+mapkp)
v5 = k5*mkkpp*mapkp/(j5+mapkp)
vm5 = km5*kpase*mapkpp/(jm5+mapkpp)

init mkkks = 0.0
init mkkp = 0.0
init mkkpp = 0.0
init mapkp = 0.0
init mapkpp = 0.0

tot3 = 3.0
tot2 = 1200.0
```

Untitled2 - Run 1: mapkpp vs. TIME

Page 1

Run ▶ ✕ 🔒 0 ⊡ F L P ⌃ ⌃ ● ⊞ ⚙ ℤ   Run 1: 8019 steps in 0.226 seconds

mapkpp vs. TIME

mkkks mkkp mkkpp mapkp mapkpp mkkk mkk mapk

Untitled2 - Sliders

☐ 10x ◀ ▶ ──┤──────────────── e1 =   0.1

☐ 10x ◀ ▶ ────┤────────────── STOPTIME = 8000

Ready

Start   Berkeley Madonna - ...   2:54 PM
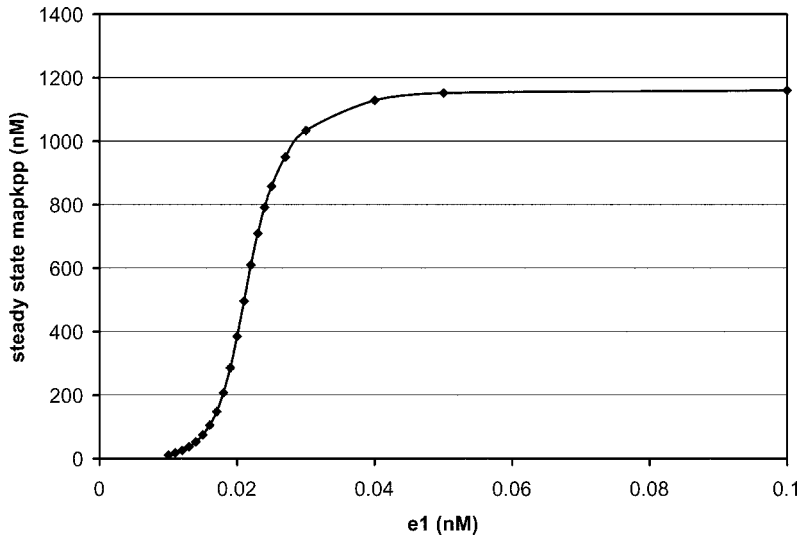
Fig. 3. Graph of steady-state value of *mapkpp* (concentration of active MAPK) as a function of signal strength (here taken as the concentration of first enzyme in the cascade, *e1*).

3.3. Steady-State Levels of Active MAPK vs Input Signal

Using the parameter sliders, we can simulate the steady-state response of the MAPK cascade (as indicated by the steady-state levels of *mapkpp*) to different strengths of signal (here taken as directly proportional to the value of the parameter *e1*, the concentration of the enzyme that catalyzes the activation of MKKK). One would repeat the run shown in **Fig. 2** for various *e1*, vary STOPTIME so that *mapkpp* has enough time to level off, and then determine the steady-state value of *mapkpp* (click on the readout button located just above the graph and drag/click the crosshair to the end of the curve to determine the steady-state value). A graph of the steady-state *mapkpp* vs *e1* is shown in **Fig. 3**, which is identical to one of Huang and Ferrell's **(1)** results (their Fig. 2) after some rescaling. This sharp sigmoidal curve corresponds to a Hill coefficient of 4.9, which demonstrates the ultrasensitivity of the MAPK cascade to changes in signals (such as *e1* here) when these are at levels within the switching region. Using parameter sliders, the user can vary as many parameters as desired to

---

Fig. 2. *(previous page)* BM simulation environment showing a graph (foreground window) that is automatically generated by clicking the "Run" button on the Equations window (background). Parameter sliders that allow the user to vary *e1* and STOPTIME are shown below the graph.

explore their influence on the dynamic and steady-state behavior of the cascade. *See* **Note 4** for other possible projects.

4. Notes

1. **Table 1** provides a short list of computer simulation software that can be downloaded from the Internet. *MCell* is a general Monte Carlo simulator that takes into account three-dimensional subcellular architecture in models of ligand diffusion and signal transduction. *MCell* has been used by its developers to simulate the microphysiology of synaptic transmission. The aim of the E-Cell project is to create a modeling and simulation environment that may allow the user to predict the dynamics of living cells through integrative models incorporating gene regulation, and metabolic and signaling pathways. To date, *E-Cell* has been used to construct a model of a hypothetical cell having a minimal gene set. Virtual Cell is another general computational framework for simulating cellular physiology and also takes into account subcellular spatial geometries, both user defined and empirically derived. Gepasi is a software package intended to simulate the kinetics of biochemical reaction networks and includes tools such as data fitting and optimization, metabolic control analysis, and linear stability analysis. SCAMP is another general purpose simulator of metabolic and chemical reaction networks; this freeware also carries out calculations involved in metabolic control analysis. Jarnac is essentially a rewrite of SCAMP with the added feature, among others, of a designer that enables users to build models visually. Another freeware is DBsolve, which provides an integrated development environment for metabolic, enzymatic, and receptor-ligand binding simulation. Cellerator is an automatic model generator that allows computerized construction of the differential equations, which is needed especially when there is a combinatorial explosion on the number of possible states of protein complexes (*see* **Note 4** on modeling involving scaffolds).

2. Implicit in the differential equations used for the Huang–Ferrell model are the assumptions that the numbers of all molecular species involved are large so that we can follow the time evolution of their concentrations in a deterministic manner (i.e., through the continuous differential equations), and that the reactions occur in a spatially homogeneous solution. In fact, both of these assumptions may not apply in actual cellular systems, and more realistic but computationally intensive calculations, such as those carried out by *MCell* (*see* **Table 1**), should be performed. Gibson and Mjolsness *(2)* provide an excellent summary of various levels of modeling biochemical networks.

3. The kinetic equations in **Table 2** differ from those used by Huang and Ferrell *(1)* in that we have assumed that the Michaelis-Menten rate expression applies to each reaction step in **Fig. 1**. Huang and Ferrell considered the detailed enzymatic mechanism—i.e., $E + S \leftrightarrow ES \rightarrow E + P$ (where E = active enzyme, S = substrate, ES = enzyme-substrate complex, and P = product), for each reaction step. If the interest is on steady-state behavior such as in **Fig. 3**, our simplification will give the same results as Huang and Ferrell's.

4. Asthagiri and Lauffenburger *(3)* have recently carried out a computational study of feedback effects, both positive and negative, on the dynamics of signal transduction. Their approach involves breaking down the complex signaling network into modules, namely the receptor-ligand processing module, the adaptor chain module, and an enzyme-cascade module (of which the MAPK cascade is an example). Negative feedback among these modules can generate signal adaptation as well as sustained concentration oscillations. Kholodenko *(4)* has utilized a quantitative measure of global sensitivity of a MAPK cascade and showed that a negative feedback lowers this sensitivity whereas a positive feedback increases it. Other factors that could significantly affect signal propagation include association between members of a MAPK cascade (e.g., *see* **ref. 5**) and the presence of scaffold proteins that bind the members of the kinase cascade; Levchenko et al. *(6)* have recently modeled the latter.
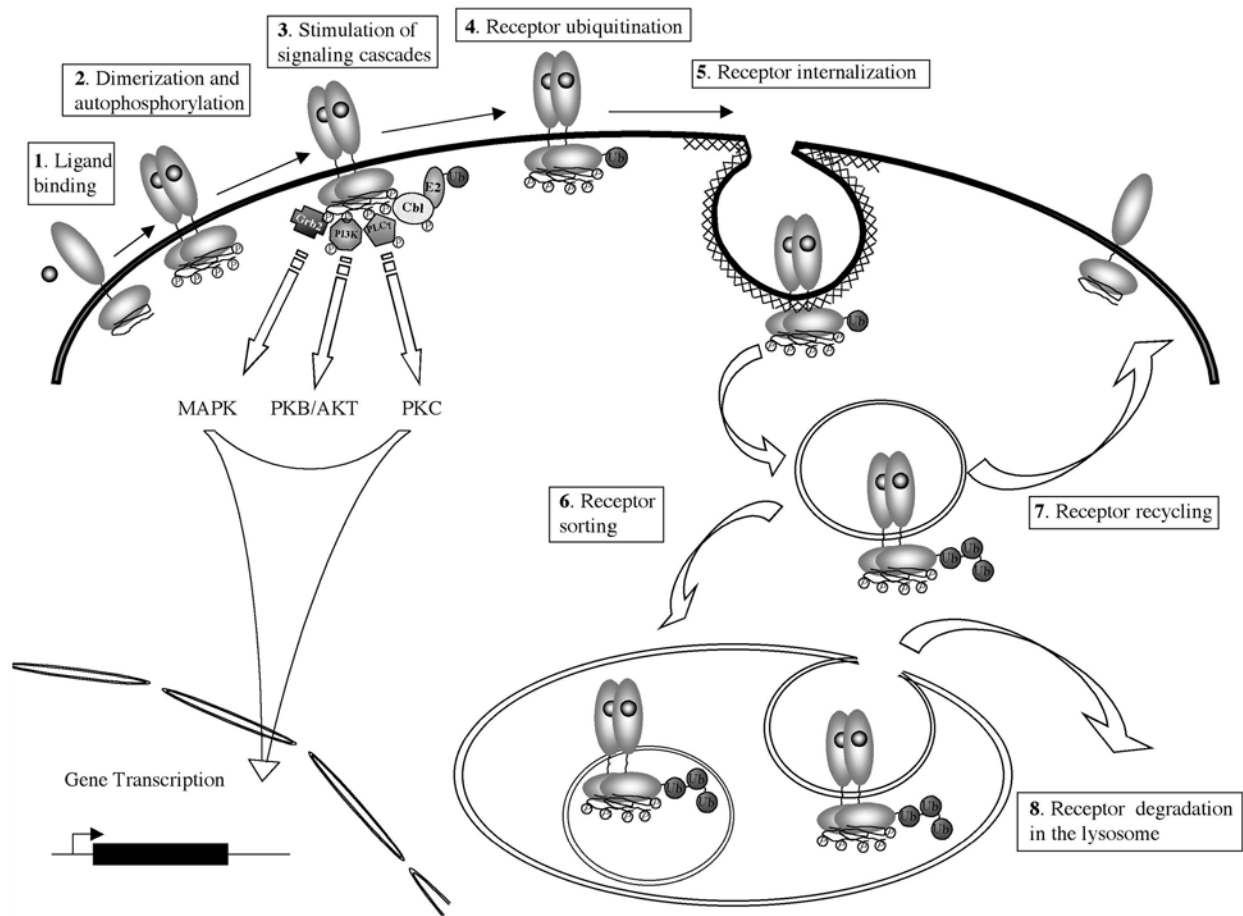
Acknowledgments

References

1. Huang, C-Y. F. and Ferrell, J. E., Jr. (1996) Ultrasensitivity in the mitogen-activated protein kinase cascade. *Proc. Natl. Acad. Sci. USA* **93,** 10,078–10,083.
2. Gibson, M. A. and Mjolsness, E. (2001) Modeling the activity of single genes, in *Computation Modeling of Genetic and Biochemical Networks* (Bower, J. M. and Bolouri, H., eds.), MIT Press, Cambridge, MA, pp. 1–48.
3. Asthagiri, A. R. and Lauffenburger, D. A. (2001) A computational study of feedback effects on signal dynamics in a mitogen-activated protein kinase (MAPK) pathway model. *Biotechnol. Prog*. **17,** 227–239.
4. Kholodenko, B. N. (2000) Negative feedback and ultrasensitivity can bring about oscillations in the mitogen-activated protein kinase cascades. *Eur. J. Biochem.* **267,** 1583–1588.
5. Cheng, J., Yang, J., Xia, Y., Karin, M., and Su, B. (2000) Synergistic interaction of MEK kinase 2, c-Jun N-terminal kinase (JNK) kinase 2, and JNK1 results in efficient and specific JNK1 activation. *Mol. Cell. Biol*. **20,** 2334–2342.
6. Levchenko, A., Bruck, J., and Sternberg, P. W. (2000) Scaffold proteins may biphasically affect the levels of mitogen-activated protein kinase signaling and reduce its threshold properties. *Proc. Natl. Acad. Sci. USA* **97,** 5818–5823.
7. Shapiro, B. E., Levchenko, A., and Mjolsness, E. (2002) Automatic model generation for signal transduction with applications to MAPK pathway, in *Foundations of Systems Biology* (Kitano, H., ed.), MIT Press, Cambridge, MA, pp. 145–162.

13

Signaling by Growth Factor Receptors

Bose S. Kochupurakkal and Yosef Yarden

1. Introduction

Growth factor receptors (GFRs) represent a subset of a large class of proteins that serve as extracellular environment sensors of the cell. They are single transmembrane proteins with an extracellular ligand-binding domain (LBD) and an intracellular tyrosine kinase domain *(1,2)*. Individual GFRs are expressed in specific cell types and their expression is highly regulated during development. In the case of polarized cells, newly synthesized receptors are targeted to specific faces of the cell through the exocytic pathway. The mechanism of signal transduction by ligand-activated GFRs is schematically depicted in **Fig. 1**. Oligomerization induced by ligand binding results in the allosteric activation of the intracellular tyrosine kinase *(3)*. A variety of cellular proteins are phosphorylated by the activated kinase, including the receptors themselves *(4)*. The phosphorylated tyrosine residues serve as docking sites for various signal transducers containing a phosphotyrosine-binding domain or an Src homology 2 (SH2) domain. Thus, a multiprotein signaling complex is assembled in the vicinity of the receptor. The ligand determines which receptors are activated, whereas the receptors involved dictate the type of proteins recruited to the signaling complex, which in turn determines the nature of the downstream signaling cascade. Although a large number of GFR families have been identified, their mode of activation, orchestration of signaling, and downregulation are modular *(5)*. As an introduction to specific methods, we elaborate on mechanisms of receptor activation, signaling, and downregulation regarding the well-characterized ErbB family of receptor tyrosine kinases (also called subtype I receptor tyrosine kinases).

2. Dimerization and autophosphorylation

3. Stimulation of signaling cascades

4. Receptor ubiquitination

5. Receptor internalization

1. Ligand binding

E2

Cbl

Ub

Grb2

PI3K

PLCγ

MAPK

PKB/AKT

PKC

6. Receptor sorting

7. Receptor recycling

8. Receptor degradation in the lysosome

Gene Transcription

1.1. ErbB Signaling Network

The mechanisms by which signaling events triggered by the ErbB receptors integrate with and alter ongoing programs within the cell is the fundamental issue. Genetic studies in *Caenorhabditis elegans, Drosophila*, and mammals have elucidated some of the mechanisms that have evolved to confer specificity to generic signals largely depending on the cellular context. Cells in culture, however, offer a useful experimental system to study molecular interactions of signaling proteins, because they circumvent the need for extremely sensitive assays by way of a large homogeneous population of cells and the ability to alter cellular equilibrium to study particular pathways.

Four receptors and a multitude of ligands are the building blocks of the ErbB signaling module *(6)*. The receptors are single transmembrane proteins with an extracellular LBD and an intracellular tyrosine kinase domain. Several tyrosine, serine, and threonine residues in the C-terminal half serve as substrates of both receptor *(7)* and nonreceptor tyrosine and serine/threonine kinases. Of the four receptors, ErbB-3 has an inactive kinase *(8)*, while no known ligand binds ErbB-2 with high enough affinity to induce homodimers *(9)*. Therefore, of the 10 possible receptor homo- and heterodimer combinations, ErbB-2 homodimers may not form and ErbB-3 homodimers are signaling impaired. However, ErbB-2 was identified as the most favorable heterodimer partner of the other receptors *(10)*. All ErbB ligands are characterized by the classic epidermal growth factor (EGF)-like fold and are expressed as transmembrane precursors on the cell surface *(11)*. Metalloproteases regulated by G protein-coupled receptors have been shown to process the ligands into active soluble peptides *(12)*.

Fig. 1. *(opposite page)* Schematic representation of receptor activation and downregulation. Ligand-induced dimerization leads to auto- and transphosphorylation of tyrosine residues in the C-terminus of the receptor, which serve as docking sites for cytoplasmic proteins containing a phosphotyrosine binding domain. Note that some of the recruited proteins also serve as substrates of the receptor. Only a few of the pathways activated by the receptor are depicted here. Many downstream effectors are subsequently activated, and the signal is carried across the nuclear membrane, culminating in nascent gene expression and cell-fate determination. The c-Cbl•E2 complex may interact with the receptor already at the cell surface, and it mediates receptor ubiquitination. Ubiquitinated receptors are then sorted into clathrin-coated pits and endocytosed. The fate of the receptor in the early endosome may be determined by the extent of ubiquitination: de-ubiquitinated receptors may be recycled back to the plasma membrane while extensively ubiquitinated receptors are apparently carried to multivesicular bodies and eventually to the lysosome, where they are degraded.

Spatiotemporal expression of receptors and ligands defines signal specificity, but binding affinity of the ligands for various receptors fine-tunes signaling when multiple receptors are in the vicinity of a particular ligand *(13)*. EGF and transforming growth factor-α bind ErbB-1, and the Neuregulin (NRG) ligands, except NRG4, bind ErbB-3 and ErbB-4. Ligands such as Betacellulin *(14)* and NRG4 *(15)* bind both ErbB-1 and ErbB-4, and Epiregulin is a pan ErbB ligand *(16)*. Ligand-specific activation of receptor subsets defines the first order in generating signal specificity *(17)*.

1.2. Signal Generation at the Cell Surface

Phosphorylation of tyrosine residues at the C-terminus of the receptors on ligand-induced dimerization is the first step of receptor activation. Sequences flanking the phosphorylated tyrosine residues determine the identity of phosphotyrosine binding domain containing proteins that will be recruited to the receptor *(18)*. ErbB-1 or ErbB-2 containing receptor complexes recruit Grb2, an SH2 and SH3 domain containing adapter protein, from the cytoplasm to the plasma membrane *(19)*. SH3 domain-mediated interaction of Grb2 and a guanine 5'-triphosphate (GTP) exchange factor, SOS, at the plasma membrane leads to the activation of mitogen-activated protein kinase (MAPK) pathway through Ras, Raf, and MEK *(20)*. Other adapter proteins such as Crk, Nck, Grb7, c-Cbl, Eps-15, and Eps-8 are recruited to the plasma membrane in a receptor-specific manner. SHC, an adapter protein containing an SH2 domain, is recruited to all the receptors and also tyrosine phosphorylated by the receptor's kinase. Phosphorylated SHC can also recruit the Grb2-SOS complex and activate the MAPK pathway. Several proteins that contain both SH2 and catalytic domains, such as phosphatidylinositol 3 kinase (PI3K), phospholipase C-γ (PLC-γ), c-Abl, Src, and phosphatases such as PTP1D and ras-GAP, are recruited in a receptor-specific manner *(21)*. For example, PI3K is directly recruited to ErbB-3, while activation of the PI3K pathway by ErbB-1 and ErbB-2 is mediated by Ras *(22)*. Another group of membrane-localized protein adapters, such as insulin receptor substrate *(23)*, fibroblast growth factor receptor substrate *(24)*, and Gab-1 *(25)*, which contain multiple tyrosine phosphorylation sites and diverse recognizable structural motifs, are recruited and phosphorylated by activated receptors. These proteins then serve as docking sites for distinct downstream effectors, and thereby contribute another layer of signal specification.

1.3. Downstream Signaling Pathways

Signaling pathways activated at the cell surface are decoded into specific cellular functions such as cell proliferation, survival, migration, and differentiation. On activation by MEK, MAPK translocates to the nucleus and acti-

vates fos, jun, and myc, a set of transcription factors, and their downstream genes that constitute the proliferative response *(26)*. MAPK may also activate transcription factors expressed in a tissue-specific manner to elicit specific responses. Another major route of signaling involves the generation of lipid messengers derived from phosphatidylinositol. For example, ErbB-3 and -4 directly recruit and activate the catalytic subunit of PI3K to the plasma membrane. Activated PI3K phosphorylates phosphatidylinositol 4' phosphate $PI(4,5)P_2$ and $PI(4)P$ to generate active second messengers. These second messengers mediate translocation of pleckstrin homology domain containing proteins such as PDK-1 and PKB to the plasma membrane *(27,28)*. Substrates of PKB include the proapoptotic protein BAD and the FKHR-1 transcription factor *(29)*. PKB has also been shown to stimulate glycogen synthase kinase and phosphofructokinase, an enzyme involved in glycolysis and glyconeogenesis. PTEN and SHIP are phosphoinositol-specific phosphatases that negatively regulate phosphoinositol-dependent signaling by dephosphorylating lipid messengers containing a phosphate group on the 3' carbon of the inositol ring *(30,31)*.

Another pathway regulated by lipid messengers involves PLC-γ, an SH2 domain containing enzyme, which translocates to the plasma membrane and hydrolyzes $PI(4,5)P_2$, thereby generating two second messengers, IP3 and diacylglycerol (DAG). IP3 activates specific receptors in the endoplasmic reticulum resulting in calcium release and activation of Ca^{+2}/calmodulin-dependent protein kinases (e.g., PYK2) and phosphatases (e.g., Calcineurin) *(32,33)*. On the other hand, DAG and calcium ions bind to and activate protein kinase C (PKC). The DAG-PKC complex translocates to the membrane, where PKC phosphorylates various proteins including receptors for growth factors, a process that initiates their inactivation *(34)*. Although only a subset of different signaling pathways that are activated by the receptor has been described, it is already evident that the kinetics and temporal pattern of activation of these pathways and their coupling to genes expressed in a cell-specific manner confers specificity to the signal *(35,36)*.

1.4. Signal Attenuation

Protein and lipid phosphatases, GTPase activating enzymes, and targeted degradation of activated kinases by the ubiquitin machinery are important components in refining the signal. Endocytosis followed by recycling or degradation of activated receptors is a mechanism used to curtail signaling from the receptors. Argos, an inhibitory ligand of the *Drosophila* EGF receptor (DER); Kekkon, a transmembrane protein that binds to DER, thereby inhibiting receptor oligomerization, Ras-GAP, a GTPase activating protein; d-Cbl, an ubiquitin E3 ligase; and Sprouty have been identified as negative regulators of the ErbB-1 signaling pathway in *Drosophila (37,38)*. Many of these genes such as Sli-1

(Cbl), GAP-1 (Ras-GAP), and others such as ARK-1, have been identified in the more evolutionarily primitive organism *C. elegans (39)*.

On ligand-induced activation, ErbB-1 is rapidly recruited into clathrin-coated pits and endocytosed *(40)*. A molecular mechanism for sorting activated plasma membrane receptors to the pits is still elusive, but lessons gained in yeast imply involvement of receptor ubiquitination *(41)*. Protein sorting to clathrin-coated invaginations of the plasma membrane is mediated by binding to adapter protein 2 (AP2), which then recruits clathrin heavy chain. Although hydrophobic motifs on the receptor have been implicated in binding to AP2, ablation of these residues does not block internalization *(42)*. Another protein that is specifically recruited and phosphorylated by some GFRs, Eps-15, may play a role in this process because it can also bind AP2 *(43)*. RalBP1, a GTP-binding protein; c-Src; and Grb2 have also been implicated in recruiting ErbB-1 to clathrin-coated pits, but the mechanisms underlying endocytosis of GFRs are still poorly understood. Nevertheless, ubiquitination of ErbB-1 and other receptors may fulfill a pivotal role in both ligand-induced and tonic internalization of the ligand-receptor complexes. Receptor ubiquitination mediated by c-Cbl has been identified as a possible modification for targeting endocytosed receptors to the lysosome *(44,45)*. c-Cbl contains a phosphotyrosine binding domain that binds a specific phosphotyrosine of ErbB-1, and a ring finger domain that binds an ubiquitin-loaded E2 enzyme (reviewed in **ref. 46**). Exactly how ubiquitinated receptors are sorted for internalization and for degradation by lysosomal hydrolases is still unclear. However, many other proteins such as PI3K, Hrs, Stam, SNX-1, and PKC, may orchestrate this highly complex process, and the subsequent desensitization of receptor's signaling.

2. Materials

2.1. Ligand-Receptor Interactions

2.1.1. Radiolabeling of EGF

The method of choice for radioiodine labeling of ligands is the use of 1,3,4,6-tetrachloro-3α, 6α-glycouril (Iodogen from Pierce, Rockford, IL).

1. Iodogen (Pierce) 1 mg/mL in chloroform.
2. EGF (1 mg/mL) (Sigma, St. Louis, MO).
3. Na^{125}I (1 mCi) (Amersham Pharmacia, Uppsala, Sweden).
4. P10 column (2 mL) (Pierce) equilibrated with 15 mL of column buffer.
5. Column buffer: phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and 500 μM dithiothreitol (DTT).

2.1.2. Determination of Binding Affinity of EGF and ErbB-1

1. Cultured cells stably expressing ErbB-1 (e.g., Chinese hamster ovary [CHO] cells transfected with the corresponding plasmid and selected for overexpression).

2. Binding buffer: 500 mL of DME/F12 medium containing 20 mM HEPES (pH 7.5) and 0.1% BSA.
3. Lysis buffer: 0.1 N NaOH and 0.1% sodium dodecyl sulfate (SDS).
4. Solution A: 87.5 μL of PBS and 17.5 μL of ^{125}I-EGF (estimated specific activity of 10^6 cpm/mL).
5. Serial dilutions of solution A (hot only): Label eight tubes from 1–8 and add 400 μL of binding buffer into each one. Add 100 μL of solution A to the first tube, mix, and transfer 100 μL from it to the next tube. Do the same for all the other tubes.
6. Solution B (hot plus cold): 12.5 μL PBS, 70 μL of EGF (100 μg/mL) and 17.5 μL ^{125}I-EGF.
7. Serial dilutions of solution B: Label eight tubes a–h and add 400 μL of binding buffer to each one. Add 100 μL of solution B to the tube marked "a," mix, and transfer 100 μL to tube "b." Do the same for all the other tubes.

2.2. Receptor Activation and Recruitment of Signaling Pathways

2.2.1. Receptor Phosphorylation and MAPK Activation

1. Seven 10-cm plates containing approx 80% confluent CHO cells stably expressing ErbB-1.
2. Five milliliters of DME/F12 medium (without serum and antibiotics) and 35 mL of DME/F12 medium (without serum and antibiotics) containing 100 ng/mL of EGF.
3. Lysis buffer: 20 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 10 mM sodium fluoride, and 2 mM phenylmethylsulfonyl fluoride (PMSF) (freshly prepared).
4. Protease and phosphatase inhibitors: 1 mM benzamidine, 2 mM sodium orthovanadate, 10 μg/mL of leupeptin, and 10 μg/mL of aprotinin.
5. HNTG buffer: 20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol.
6. Ice-cold PBS.
7. Stripping buffer: 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7.
8. Antimouse IgG coupled to agarose beads, monoclonal antibodies (MAbs) specific to ErbB-1, as well as antibodies against MAPK, active-MAPK, and antiphosphotyrosine antibodies.

2.2.2. Recruitment of Substrates

1. Two 10-cm plates of CHO cells (approx 80% confluent).
2. Constructs encoding ErbB-1, or various mutants of the receptor (e.g., a kinase-defective receptor and phosphorylation site mutants); c-Cbl; and an empty vector (pCDNA3).
3. Lysis buffer containing protease inhibitors and HNTG buffer.
4. Serum-free F12 medium.
5. Lipofectamine for transfection of cultured cells.
6. Goat antimouse antibody covalently coupled to agarose beads.
7. Antibodies against ErbB-1, c-Cbl, and ubiquitin.

2.3. Receptor Internalization, Ubiquitination, and Downregulation

Receptor endocytosis and degradation are determined by measuring the amount of receptors at the cell surface usually using radiolabeled ligand.

2.3.1. Receptor Downregulation

1. Expression vectors encoding ErbB-1 (wild-type, WT), a kinase-defective mutant (Kin-), tyrosine 1045 mutated to phenylalanine (Y1045F), and c-Cbl.
2. Subconfluent 10-cm dish of CHO cells.
3. Solution A: 15 mL binding buffer and 15 µL EGF (100 µg/mL).
4. Solution B: 15 mL of binding buffer and 35 µL of ^{125}I-EGF (final concentration is 1–5 ng/mL).
5. Binding buffer: DMEM/F12, 20 mM HEPES (pH 7.5) and 1% BSA.
6. Acetic acid wash buffer: 0.15 M acetic acid and 0.15 M NaCl.
7. Lysis buffer: 0.1 N NaOH and 1% SDS.

2.3.2. Receptor Ubiquitination Assay

1. Cells overexpressing ErbB-1 (e.g., A431 or HER-14 cells).
2. Anti-ErbB-1 antibodies and antimouse agarose.
3. Lysis buffer: 20 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 10 mM sodium fluoride, and 2 mM PMSF (freshly prepared).
4. RIPA buffer: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 0.5% Na-deoxycholate.
5. Rabbit reticulocyte lysate (Promega, Madison, WI).
6. GST-Cbl and GST-Shc (recombinant: expressed in *Escherichia coli*).
7. Ubiquitination wash buffer: 40 mM Tris-HCl (pH 7.5), 5 mM MgCl$_2$, and 2 mM DTT.
8. Ubiquitination reaction buffer: 1.6 µL of Tris-HCl (pH 7.5) (1 M), 2 µL of MgCl$_2$ (0.1 M), 0.8 µL of DTT (0.1 M), 0.8 µL of ATPγS (0.1 M), 5 µL of rabbit reticulocyte lysate, 4 µL of ^{125}I-ubiquitin (3 µg/mL). Add 5 µL of GST-Cbl/GST-Shc (1 mg/mL), 0.4 mg/mL of unlabeled ubiquitin or 8 µL of ATP (0.1 M) to the respective tubes and make up the volume to 40 µL with deionized water.

2.3.3. Immunofluorescence Studies

1. CHO cells.
2. Plasmids encoding ErbB-1-GFP (EGFP-N3; Clontech) and HA-tagged c-Cbl (pCDNA3; Invitrogen, Groningen, Netherlands).
3. Texas Red–EGF (Molecular Probes, Eugene, OR), rat anti-HA antibody (Roche Diagnostics GmbH), and donkey antirat-CY3 (Jackson, Bar Harbor, ME).
4. Cover slips (autoclaved) and glass slides (Corning).
5. Fixing buffer: PBS containing 4% paraformaldehyde.
6. Wash buffer (PBS-T): PBS containing 0.2% Triton X-100.
7. Blocking solution: 2% goat serum, 0.5% BSA in PBS-T.
8. Antibody dilution buffer: PBS-T containing 0.2% BSA.
9. Mowiol 4-88 (Calbiochem) for mounting the slides.

2.4. Determination of Transcriptional and Mitogenic Responses to Growth Factors

2.4.1. Transcription Activation Assays

1. Luciferase vector (Clontech) containing cytomegalovirus (CMV) promoter (positive control), Fos promoter, and the serum response element (SRE).
2. ErbB-1-expressing cells (e.g., CHO cells stably expressing ErbB-1, CB1 cells).
3. Lipofectamine (Life Technologies).
4. 5X Lysis buffer (Promega).
5. D(–)-Luciferin (Roche diagnostics GmbH) stock solution: Dissolve 10 mg of luciferin in 3.75 mL of 10 mM Tris-acetate (pH 7.8). Store as 100-mL aliquots in –20°C protected from light.
6. O-Nitrophenyl-β-D-galactopyranoside (ONPG) (Sigma) stock solution: 100 mM sodium phosphate containing 4 mg/mL of ONPG.
7. Luciferase buffer: 100 mM Tris-acetate (pH 7.8), 10 mM Mg-acetate, 1 mM EDTA (pH 8.0).
8. Luciferase reagent buffer: 13.5 mL Luciferase buffer, 100 μL of 10 mM Luciferin, 150 μL of 200 mM adenosine triphosphate (ATP) (prepared in deionized water). Store at –20°C and protect from light.
9. 100X Mg solution: 100 mM MgCl$_2$, 4.5 M β-mercaptoethanol. Store at –20°C.
10. Sodium phosphate buffer (pH 7.5): 41 mL of 200 mM Na$_2$HPO$_4$, 9 mL of 200 mM NaH$_2$PO$_4$, and 50 mL of water.

2.4.2. Mitogenic Assays Using Interleukin-Dependent Cells

1. The murine hematopoietic progenitor cell line 32D.
2. RPMI-1640 medium supplemented with antibiotics, 10% heat-inactivated fetal bovine serum, and interleukin-3 (IL-3) (commercial preparation or conditioned medium form producer cells like X63/0 cells).
3. RetroPack PT67 Cell Line (Clontech).
4. pLXSN plasmid encoding ErbB-1.
5. Polybrene (Sigma) stock solution: 8 mg/mL of polybrene in PBS.

3. Methods

3.1. Ligand-Receptor Interactions

Studies on ligand-ErbB interactions have mainly focused on receptors involved in oligomerization induced by a particular ligand, and binding affinity of different ligands for homo- or heterodimers of the receptors. Ligand affinity values determine which receptors will dimerize when a ligand is applied to cells expressing different receptors of the same family. The receptors involved in dimerization can be identified either by coimmunoprecipitation or by crosslinking the ligand-receptor complex followed by immunoprecipitation and Western blotting with relevant antibodies. Affinity values have been determined using cells either stably or transiently expressing the receptor. Alternatively, soluble receptor extracellular domains fused to the Fc fragment
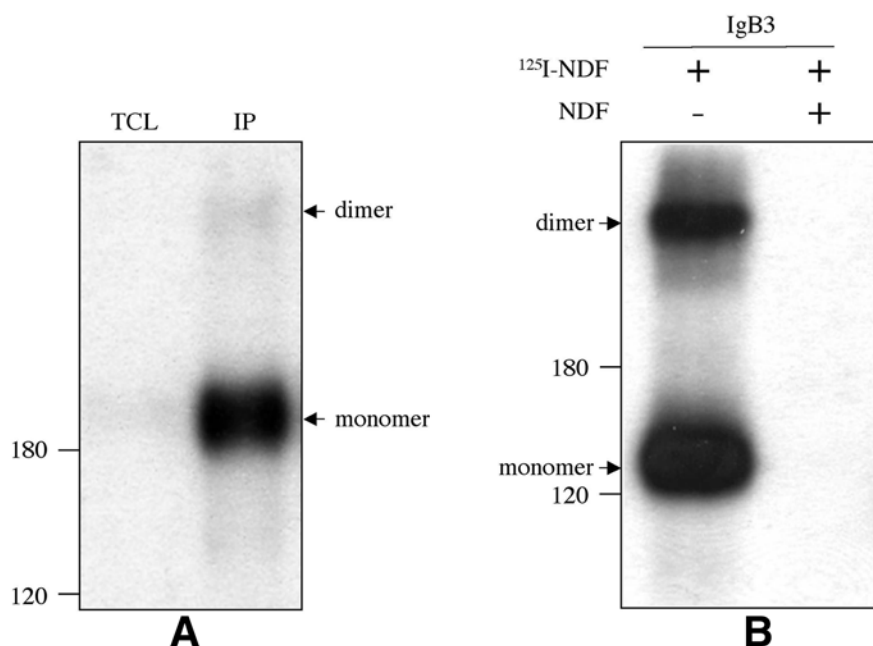
Fig. 2. Crosslinking of radiolabeled NDF to ErbB-3. **(A)** CHO cells stably express-ing ErbB-3 were harvested in PBS and incubated on ice with ^{125}I-NDF (10 ng/mL) for 1 h and then treated with BS^3 (1 mM), a homobifunctional crosslinking agent, for 30 min. The cells were washed gently with PBS, lysed, and ErbB-3 was immunoprecipi-tated using an MAb against the extracellular domain. The immunoprecipitate (IP) and 50 µL of the total cell lysate (TCL) were separated on an SDS-PAGE, and the dried gel was exposed to an autoradiogram. **(B)** The extracellular domain of ErbB-3 was fused to the Fc fragment of human IgG and expressed as a secreted fusion protein (IgB3). The fusion protein was affinity purified using protein A Sepharose from conditioned medium and a crosslinking assay was performed. Equal amounts of the receptor's extracellular domain bound to beads were incubated with radiolabeled NDF (50 ng/ mL), either in the presence or in the absence of excess unlabeled NDF (50-fold), for 1 h and further incubated with BS^3 for 30 min. The beads were extensively washed, then boiled in sample buffer under reducing conditions, and the protein was separated using SDS-PAGE and exposed to an autoradiogram.

of immunoglobulins may be constructed and their binding characteristics assayed using immunologic methods *(47)*, analytical centrifugation *(48)*, or plasmon resonance *(49)*. Recombinant extracellular domains of the receptors have also been used in titration calorimetry and small-angle X-ray scattering experiments to determine stoichiometry of ligand-receptor interactions *(50)*. An example of ligand crosslinking to soluble, recombinant ErbB-3, as well as

a cellular transmembrane form of this receptor, is presented in **Fig. 2**. Finally, crystal structure studies of ligand-receptor complexes help reveal the molecular details of this interaction *(51–55)*. A method for radiolabeling of EGF and determining the binding affinity of EGF and ErbB-1 is described (*see* **Note 1**).

3.1.1. Radiolabeling of EGF

1. Add 50 µL of the Iodogen solution to the bottom of a microfuge tube, and leave it in a ventilated hood until the chloroform evaporates and a thin coat of Iodogen is left on the wall. These tubes can be prepared in advance and stored at –20°C.
2. Add 45 µL of PBS, 5 µL of EGF, and 1 mCi of $Na^{125}I$ to the Iodogen-coated tube, and incubate on ice for 30 min with intermittent shaking.
3. Add 150 µL of PBS to the tube, mix with a pipet, remove a sample (5 µL, for calculation of specific activity), and load the rest of the reaction mix on the drained column.
4. After the reaction mix enters the column, load two aliquots of 200 µL of column buffer in series to ensure that the sample enters the column as a tight band, and then load 2 mL of column buffer. Flow of the radiolabeled ligand through the column can be monitored using a Geiger counter.
5. Collect 500 µL fractions. The ligand is normally present in the second fraction and the first 300–350 µL of the third.
6. Calculate the specific activity of the radiolabeled EGF by performing trichloro-acetic acid precipitation assay on samples collected before and after separation on the column. BSA (1 mg/mL) may be used as a carrier in this assay.

3.1.2. Determination of Binding Affinity of EGF and ErbB-1

1. Trypsinize CHO cells stably expressing ErbB-1, and plate equal number of cells in each well of a 48-well plate 24 h prior to the experiment.
2. On the day of the experiment, transfer the plate to ice, aspirate the medium, and wash the cells once with ice-cold binding buffer.
3. Add 100 µL from each tube of serial-diluted solution A to the cells in the top three rows and 100 µL of serial-diluted solution B to the bottom three rows such that cells in the same column have the same concentration of radiolabeled ligand. The experiment is done in triplicates.
4. Incubate the plate on ice for 2 h.
5. Aspirate the medium from the wells, and wash the cells with 0.5 mL of ice-cold binding buffer (twice).
6. Add 200 µL of the lysis buffer to each well and incubate at room temperature (20 min). Collect the lysates in radiation counting tubes and determine the counts in each tube. This value corresponds to bound ligand.
7. Count the radioactivity in 100 µL of each of the serial dilutions. This value is the total count for each of the serial dilutions.
8. Subtract the counts obtained for bound ligand in the presence of excess unlabeled ligand (solution B) for each of the serial dilutions from the total count of the corresponding dilutions to yield the values for specific binding. Both saturation curves and a Scatchard plot can be derived from these values.

3.2. Receptor Activation and Recruitment of Signaling Pathways

Receptor activation is exemplified by ligand-induced oligomerization and receptor autophosphorylation. Western blotting immunoprecipitates of the receptor with an antiphosphotyrosine antibody such as pY20 (BD-Transduction, San Diego, CA) or 4G10 (Upstate Biotechnology, Lake Placid, NY) are used to identify receptor phosphorylation. Likewise, MAPK activation by GFRs is a common route of signaling. Temporal variations in MAPK activation by distinct receptors or stimulating ligands of the same receptor, often in a cell-specific manner, has been reported. The simplest way to detect MAPK activation is using antibodies specific to the doubly phosphorylated MAPK (Sigma) *(56)*. An alternative approach is to perform a kinase assay using immunoprecipitates of total MAPK using myelin basic protein as the substrate. Methods to determine ErbB-1 phosphorylation, MAPK activation, and substrate recruitment induced by EGF are described (*see* **Notes 2** and **3**),

3.2.1. Receptor Phosphorylation and MAPK Activation

1. Wash 105 μL of a 50% slurry of agarose beads coupled to antimouse immunoglobulins by using HNTG buffer. Add 15 μg of the anti-ErbB-1 antibody to the beads and incubate at 4°C for 1 h.
2. Prewarm DME/F12 with and without EGF to 37°C.
3. Label the plates 0, 5, 10, 15, 30, 45, and 60, indicating time of incubation with ligand.
4. Aspirate the medium from the plates and quickly add 5 mL of prewarmed medium without the ligand in the plate marked 0 and place it on ice. Add 5 mL of the prewarmed EGF-containing medium to the other plates, and transfer them to a 37ºC incubator.
5. Wash the zero time point plate with ice-cold PBS (three times), add 800 μL of lysis buffer containing inhibitors, collect the lysate in a microfuge tube using a cell scraper, and place it on ice.
6. Remove the plates from the incubator at each time point and place them on ice. Quickly process the plates as in **step 5**.
7. Incubate each cell lysate on ice for 15 min with intermittent mixing.
8. Spin the total cell lysate at 14,000g for 10 min, and transfer the supernatant into another tube.
9. Vortex the supernatants and perform a Bradford assay to ensure equal load of proteins on the gel and in immunoprecipitation reactions.
10. Collect 40 μL of the supernatant from the sample containing the least amount of protein and respective volumes from the other samples. Add concentrated (6X) gel sample buffer and heat the samples (95ºC) for 5 min. This is the total cell lysate.
11. Wash the antimouse agarose beads incubated with anti-ErbB-1 three times with HNTG buffer, and resuspend the beads in 650 μL of HNTG.
12. Aliquot 100 μL of anti-ErbB-1 antibody coupled to antimouse beads into seven microfuge tubes containing equal amounts of total cell lysate (as determined by using the protein assay).

13. Leave the tubes shaking at 4°C for 1 h.
14. After incubation, spin the beads down, aspirate the supernatant, and wash the beads three times with HNTG buffer taking care not to lose beads during aspiration. To reduce nonspecific precipitation, the resuspended beads may be transferred to a new tube just before the last wash.
15. Add 50 μL of gel sample buffer to the tubes and heat the samples as above. This is the ErbB-1 immunoprecipitate.
16. Separate the proteins by SDS-polyacrylamide gel electrophoresis (PAGE) (Bio-Rad minigel with 10 lanes), and electrophoretically transfer the proteins onto a nitrocellulose membrane.
17. Western blot a gel containing the total cell lysate with the anti-active MAPK antibody, and detect the gel containing the ErbB-1 immunoprecipitates with an antiphosphotyrosine antibody.
18. To confirm that equal amounts of protein from each sample were loaded on the gel, strip the antibodies on the membranes by incubating the membrane in stripping buffer for 30 min at 50°C. Then extensively wash the blots with TBS-T and reprobe with the general-MAPK antibody and an anti-ErbB-1 antibody, respectively.

3.2.2. Recruitment of Substrates

The simplest method for identifying proteins that interact with the receptor is coimmunoprecipitation and Western blotting for a given list of substrates. This technique is most suitable for proteins that directly interact with the receptor, but it may be unable to detect secondary and tertiary associated proteins, largely owing to the stoichiometry of the complexes. Moreover, because a large number of proteins will be precipitated nonspecifically, it is imperative that the precipitates be washed extensively, resulting in the disruption of secondary interactions. A protocol describing the elucidation of the dependence on phosphorylation of a specific carboxy-terminal tyrosine residue of ErbB-1 for its interaction with c-Cbl is presented *(45)*.

1. One day before transfection, split the CHO cells at 1:6 to prepare 12 plates.
2. Cell transfection:
   a. Label six sterile polystyrene tubes according to the expression vectors to be used (e.g., WT, WT+Cbl, Dc214, Dc214+Cbl, Kin⁻, and Kin⁻+Cbl). Note that Dc214 is a receptor mutant lacking 214 carboxyl terminal amino acids *(57)*.
   b. Add 2 μg of the respective plasmids to the tubes and make up the volume to 800 μL with F12 medium. Add 2 μg of the pCDNA3 vector to the tubes that do not contain the plasmid encoding c-Cbl.
   c. To a fresh tube add 4.63 mL of F12 medium and 170 μL of Lipofectamine and mix gently using a pipet.
   d. Add 800 μL of the Lipofectamine/F12 mixture into each of the six tubes, mix gently, and incubate at room temperature for 30 min. This is the transfection mixture.

    e. Label the plates containing CHO cells similar to the tubes in duplicates and wash the cells with F12 medium.

    f. Make up the volume of the transfection mix to 8 mL using F12 medium. Gently lay 4-mL aliquots on the cells in each of the duplicate plates.

    g. Return the plates to the incubator and replace the medium with DME/F12 medium containing 10% fetal calf serum and antibiotics after 4 to 5 h.

3. About 45–48 h after transfection, stimulate one plate from each of the duplicates with DME/F12 medium containing EGF (1–100 ng/mL), and the other with DME/F12 medium containing no EGF, for 10 min. Harvest the cells as described previously.

4. Immunoprecipitate ErbB-1 from the lysates after they are normalized for protein amounts, and separate the immunocomplexes by SDS-PAGE. Transfer the proteins onto a membrane.

5. Cut the membrane into two parts just above the 120-kDa marker. Probe the upper part of the gel with an antiubiquitin antibody, strip and reprobe with an anti-ErbB-1 antibody that recognizes the carboxyl-terminus of the receptor. Probe the lower part of the membrane with an anti-c-Cbl antibody.

3.3. Receptor Internalization, Ubiquitination, and Downregulation

The aim of these assays is to determine the kinetics of receptor endocytosis and degradation. Essentially, the amount of receptors at the cell surface at various time points, starting from the initial point of ligand addition, is determined using a radiolabeled ligand. The readouts obtained at each time point reflect the number of receptors that was not endocytosed, as well as receptors that were recycled back to the cell surface. An assay to determine the kinetics of the loss of ErbB-1 (WT), a kinase-defective mutant, and a mutant that does not bind c-Cbl (Y1045F) is described based on a previously described procedure *(44)*. **Figure 3** depicts the results of a simple downregulation assay performed on transfected CHO cells. In addition, a protocol for determination of receptor ubiquitination, a prerequisite for endocytosis of GFRs, is also described, along with a method to follow endocytosis by immunofluorescence.

Receptor ubiquitination can be studied using a cell system in which the receptor, c-Cbl, and HA-tagged ubiquitin are coexpressed in cells. Western blotting membranes containing receptor immunoprecipitates by using either antibodies to ubiquitin or an anti-HA antibody is the simplest way to detect receptor ubiquitination. **Figure 4** is an example of results obtained by using a peptide-tagged ubiquitin coexpressed with ErbB-1 and c-Cbl. Variations of the protocol include using myc-tagged ubiquitin and preincubating cells with lysosomal inhibitors such as chloroquine and/or proteasomal inhibitors *(58)*. The inhibitors prevent receptor degradation, thereby enhancing the signal of ubiquitinated receptor. Receptor ubiquitination can also be carried out in a cell-free system using immunoprecipitated receptors and rabbit reticulocyte lysate.
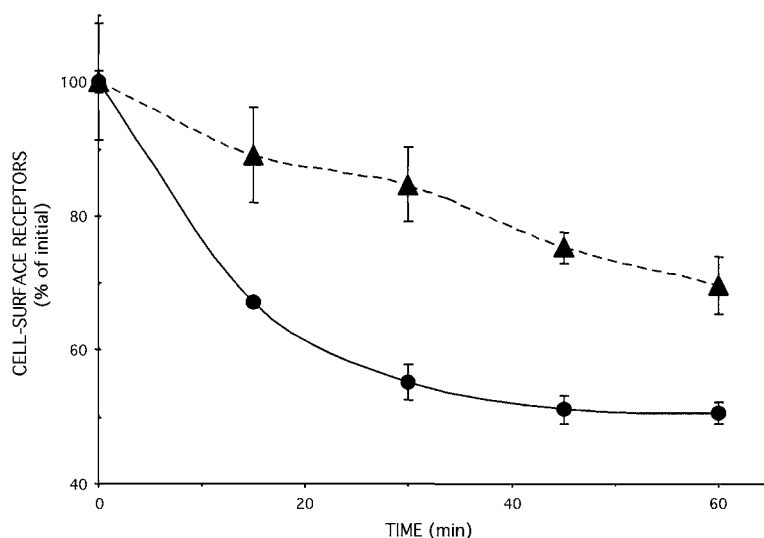
Fig. 3. Cbl-induced downregulation of ErbB-1. CHO cells were cotransfected with ErbB-1 and either an empty vector (triangles) or a plasmid encoding c-Cbl (circles). Cells were stimulated with EGF (100 ng/mL) for the indicated periods of time 48 h after transfection. The amount of receptor remaining on the cell surface was determined by removing surface-bound ligand and performing a binding assay. The experiment was performed in triplicates, and the radioactivity associated with surface receptor is plotted as a fraction of the initial amount of receptors at the cell surface.

Alternatively, recombinant E1, E2, and c-Cbl expressed in *E. coli (44)* may be used. However, since the efficiency of the in vitro reaction is not high, it is recommended that ubiquitin be radiolabeled.

Immunofluorescence studies of GFR signaling can be instructive and help establish results obtained in biochemical experiments. **Figure 5** represents the results obtained by fluorescent labeling of EGFR and c-Cbl in EGF-stimulated cultured cells. GFP-tagged ErbB-1 and fluorescent-labeled EGF have been extensively used to study receptor activation and trafficking through the endocytic pathway *(59,60)*. Fluorescence resonance energy transfer microscopy was employed to detect interactions of receptors with Grb2, an SH2 domain-containing protein that binds to phosphorylated tyrosine residues of ErbB-1 *(61)*.

3.3.1. Receptor Downregulation

1. Split the plate of subconfluent CHO cells 1:6.
2. After 12 h, select three plates and label them WT, Kin-, and Y1045F and, similarly, the other three plates including +Cbl.
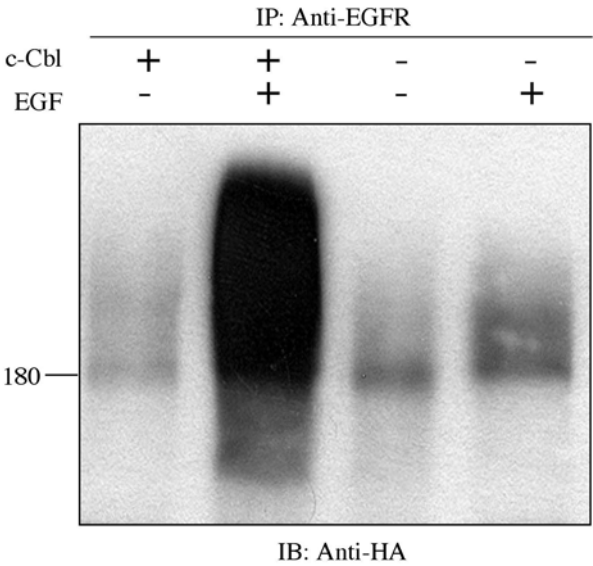
Fig. 4. c-Cbl mediates ubiquitination of ErbB-1. CHO cells transiently expressing ErbB-1, HA-tagged ubiquitin, and c-Cbl, as indicated, were stimulated with or without EGF (100 ng/mL) for 10 min at 37°C. The cells were then transferred to ice, quickly washed with cold PBS, and ErbB-1 was immunoprecipitated from the cell lysate. The immunoprecipitates were washed extensively, boiled in sample buffer, and separated by SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and probed with an anti-HA antibody.

3. Cotransfect 1 μg of each plasmid encoding the receptor either with 1 μg of empty vector or with 1 μg of the plasmid encoding c-Cbl using the Lipofectamine method described earlier.

4. Trypsinize the transfected cells 24 h after transfection, and plate an equal number of each cell type in triplicates down the column in a 24-well plate and make seven similar plates.

5. Label the plates 0, 15, 30, 45, 60, 90, and 120 corresponding to the time they will sit in the incubator with the ligand, and place the plates in the incubator.

6. After 24 h, wash the plate labeled 120 with 0.5 mL of prewarmed binding buffer, add 200 μL of solution A, and return it to the incubator. Also set a timer for 30 min.

7. Shortly before 30 min, treat the plate labeled 90 as in **step 6**, and set the timer for another 30 min.

8. Shortly before the timer goes off, treat the plate labeled 60 min as in **step 6**, and set the timer for 15 min.

9. Process the plates labeled 45, 30, and 15 as described in **step 8**, in each case setting the timer for an additional 15 min.

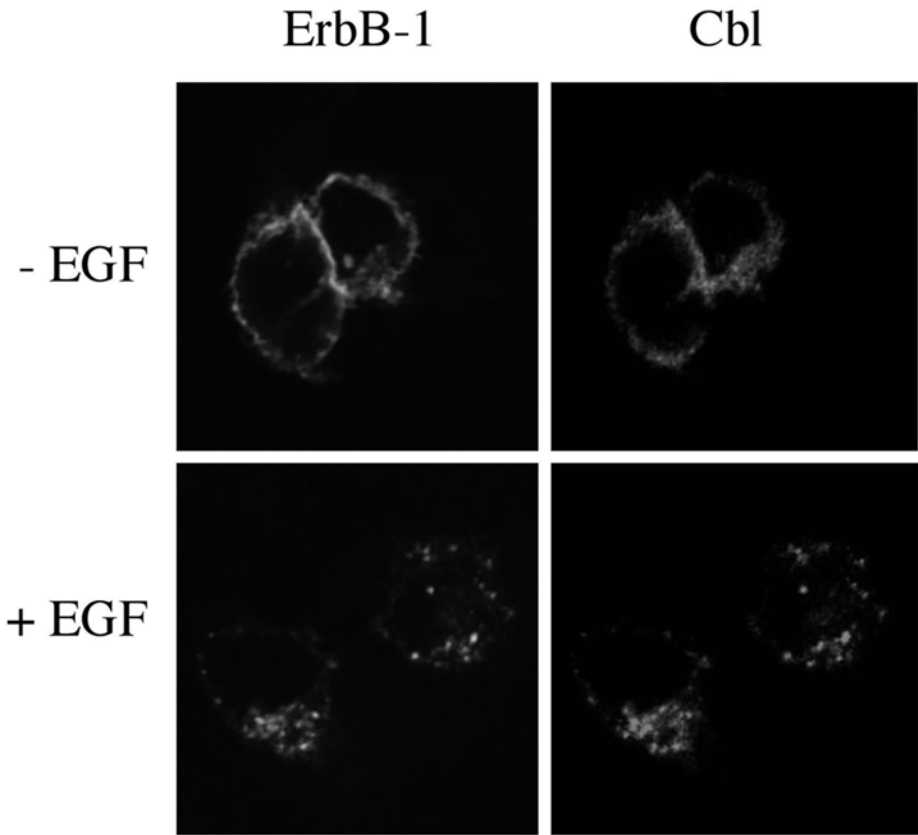## ErbB-1   Cbl

Fig. 5. c-Cbl colocalizes with the receptor in vesicular structures. CHO cells were transiently transfected with plasmids encoding ErbB-1 fused to the green fluorescent protein and HA-tagged c-Cbl, and replated on cover slips 24 h posttransfection. The cells were stimulated with or without EGF (100 ng/mL) the next day for 10 min at 37°C, transferred to ice, quickly washed with cold PBS, and fixed using paraformaldehyde. The cells were then permeabilized and incubated with anti-HA antibody for 1 h, washed and incubated with CY3-labeled donkey antibodies to rat immunoglobulins for another hour. Thereafter, the cells were washed and mounted on slides using Mowiol. The prepared slides were observed under a confocal microscope.

10. Once the incubations for all the plates are finished, transfer all of the plates to ice including the plate labeled 0. All further steps are done on ice.
11. Wash the plates with 0.5 mL of ice-cold binding buffer.
12. To remove the unlabeled ("cold") ligand bound to surface receptors, add ice-cold acetic acid wash buffer to the cells and incubate on ice for 3 min.
13. Aspirate the acetic acid wash buffer completely and add 200 μL of solution B.

14. After 2 h of incubation on ice, wash the cells with ice-cold binding buffer twice and add 0.5 mL of lysis buffer to the cells.
15. Transfer the lysates to gamma-counting tubes.
16. Take the counts obtained for the plate labeled 0 as 100% and convert the values for the other time points to percentages with respect to this value. Finally, plot the numbers as a function of time.

3.3.2. Receptor Ubiquitination Assay

The protocol describes ErbB-1 ubiquitination in a cell-free system using rabbit reticulocyte lysate.

1. Immunoprecipitate ErbB-1 from cleared cell lysates of four 10 cm subconfluent plates of A431 cells as described previously. Note that cell solubilization buffer contains no ionic detergents.
2. Wash the immunoprecipitate three times with lysis buffer and equilibrate with ubiquitination wash buffer.
3. Aliquot the immunoprecipitate equally into four tubes labeled ACU (for ATP, GST-Cbl, and ubiquitin), C, AS (S for Shc), and AC.
4. Prepare the ubiquitin reaction buffers according to labels on the tubes, and incubate the beads in reaction buffer for 1 h at 30°C.
5. Wash the beads three times with RIPA buffer, separate on an SDS-PAGE, and expose the dried gel to an X-ray film.

3.3.3. Immunofluorescence

A method to follow the fate of ErbB-1-GFP stimulated with Texas Red–EGF and cotranslocation of HA-tagged c-Cbl and the activated receptor is described.

1. Transfect an ~60% confluent plate of CHO cells with 2 μg of plasmid encoding ErbB-1-GFP and HA-Cbl.
2. Place the sterilized cover slips in two wells of four six-well plates. Trypsinize the transfected cells and seed them on the cover slips 16–20 h after transfection (use all the cells and plate them equally).
3. Thirty-six hours posttransfection, wash the cells three times with serum-free medium, and serum starve the cells for 12 h.
4. Label the plates 0T, 0C, 10T, and 15C indicating the time of incubation with EGF and the protein that is stained. There will be two wells with the same treatment.
5. Forty-eight hours after transfection, aspirate the medium from the cells, transfer the plate labeled 0T to ice, and incubate with cold medium containing Texas Red–EGF for 1 h. Stimulate plates labeled 10T and 15C with Texas Red–EGF (10 min) and EGF (15 min), respectively, at 37°C.
6. After the incubation with ligand, transfer all the plates to ice and wash the cover slips with ice-cold PBS (twice), and incubate with fixing buffer for 15 min. Note that all the procedures from this stage until after adding the secondary antibody

can be done in the same six-well plate. Alternatively, the cover slips can be transferred to a holder and immersed sequentially into buffer chambers containing different buffers (it is important to note which side of the cover slip contains the cells). Addition of buffers must be done gently through the sides of the well because there is a threat of losing or mutilating the cells.

7. Wash the cells extensively with cold PBS-T to remove all traces of paraformaldehyde. Note that Tween-20 also serves to permeabilize the cells. Cells stimulated with Texas Red–EGF are ready for mounting.
8. Carefully pick the cover slip from the well, drain it on a piece of soft paper, and mount the cover slips with Mowiol on object glasses with cells facing down.
9. Incubate the cover slips labeled 0C and 15C in blocking buffer for 1 h, wash with PBS-T, and incubate with rat anti-HA MAb (primary antibody) for 1 h.
10. Dilute the donkey antirat CY3 antibody (secondary antibody) 1:500 in blocking buffer, and spin the solution for 15 min at 14,000 rpm to precipitate aggregates that may give rise to spurious signals.
11. Incubate the cover slips with the secondary antibody for 1 h after incubating them with the primary antibody and extensively washing with PBS-T.
12. Wash the cover slips with PBS-T, carefully remove them from the plate, and mount them on slides using Mowiol with the surface containing cells facing down.
13. Set aside the slides in a dark place to dry (overnight). They can be viewed under a fluorescence or confocal microscope.

3.4. Determination of Transcriptional and Mitogenic Responses to Growth Factors

Signaling cascades triggered by activated receptors culminate in the activation of transcription factors resulting in gene expression. Methods such as DNA chip analysis hold great promise for cataloging all genes controlled by specific growth factors *(62,63)*, and promoter trap experiments have helped identify specific genes downstream to extracellular signals *(64)*. The luciferase assay has been extensively utilized to identify actual binding motifs in regulatory sequences of genes whose expression is controlled by growth factors. This assay also helps identify different signaling pathways that activate the same set of genes. Gel shift assays using specific radiolabeled promoter sequences have been used to identify different transcriptional elements that bind to these regulatory sequences. For a review on the regulation of transcription by growth factors, *see* **ref. 65**.

Another long-term assay, reflecting the cumulative effect of different signaling pathways including nuclear events, follows the mitogenic response. Preferably, the assay is performed on natural cell lines expressing the receptor of interest. Usually, transformed cells, whose multiplication cycle is relatively short, are not suitable for mitogenic assays. However, primary cells or other nonmalig-

nant cell lines yield satisfactory sensitivity. One way to circumvent the cellular environment and also test specific receptor mutants is to genetically reconstitute ligand-receptor interactions in growth factor-dependent cells. This is usually achieved in hematopoietic cell lines such as Mo7e, TF-1, 32D, and BaF3 cells. Because myeloid and lymphoid cells usually do not express ErbB receptors, such cell lines have been particularly useful for the analyses of receptor-receptor interactions in the ErbB family *(66–68)*. Because these cells are nonadherent, and the efficiency of gene transfer by transfection is relatively low with such cells, infection with retroviral vectors has been the alternative method of choice. Retrovirus-mediated introduction of DNA essentially involves two steps: first, the viruses need to be produced in special cells called packaging cells; and, second, target cells need to be infected with the viruses and stable clones selected using a selection marker (e.g., neomycin). A variety of reagents are available from suppliers such as Clontech. Use of one of the retroviral vectors called pLXSN to generate a 32D cell line stably expressing ErbB-1 is described.

3.4.1. Transcription Activation Assays

Activation of the Fos promoter and the SRE downstream to ligand-stimulated ErbB-1 is described (*see* **Note 6**).

1. Split one 10-cm plate of CB1 cells equally into fourteen 60-mm dishes and label them 1–14.
2. Cotransfect all plates with 12 μg of β–gal reporter plasmid along wit 24 μg of control luc plasmid in plate 1; 4 μg of CMV-luc plasmid in plate 2; 24 μg of Fos-luc plasmid in plates 3–8; and 24 μg of SRE-luc plasmid in plates 9–14 using the Lipofectamine method described earlier.
3. Label three plates from the set of six containing the Fos-Luc and SRE-luc plasmid; FL and SL, respectively, and the other three plates, Fβ and Sβ, respectively. The luciferase and the β-gal assays are done in triplicates. Twenty-four hours after transfection, wash the cells and add DME-F12 medium containing 10% serum and EGF (50 ng/mL).
4. After 16–18 h, harvest the cells in PBS (by using a rubber policeman) into tubes marked appropriately.
5. Luciferase assay:
   a. Incubate the tubes labeled 1, 2, FL, and SL in lysis buffer for 10 min, flicking them intermittently.
   b. Spin the cells at 14,000 rpm for 20 s and transfer 20 μL of the viscous cell extract into luminometer tubes.
   c. Add ~100 μL of luciferase reagent buffer, and read the light units in a luminometer.
6. β-gal assay:
   a. Place the tubes labeled Fβ and Sβ in a dry ice ethanol bath for 5 min and then at room temperature for 5 min, and repeat this procedure (five times) to lyse the cells.

     b. Spin the tubes at 14,000 rpm for 10 min, transfer 30 µL of the supernatant into fresh tubes, and place on ice.

     c. Add 3 µL of 100X Mg solution, 66 µL of 1X ONPG, and 201 µL of 100 m*M* sodium phosphate buffer (pH 7.5), and incubate at 37°C for 30 min or until a faint yellow color develops.

     d. Stop the reaction by adding 500 µL of 1 *M* Na$_2$CO$_3$ to each reaction, and read the optical density (OD) of the reactions at a wavelength of 420 nm (the linear range is 0.2–0.8 OD). This value gives an estimate of transfection efficiency for each plate.

7. To plot the graph, value of luciferase activity in plate 1 is subtracted from that obtained from plates 2–14. These subtracted values are then normalized based on the β-gal activity values to obtain the actual values.

3.4.2. Mitogenic Assays Using Interleukin-Dependent Cells

1. Because the PT67cells have a very short doubling time, split them 1:15 before the plate is confluent.
2. Transfect the PT67 cells at ~40% confluence with the pLXSN-ErbB-1 vector using the Lipofectamine method as described above.
3. The viral titer reaches maximum at ~48 h posttransfection. Collect the culture medium ~60 h posttransfection.
4. Assess viral titer in the supernatant by infecting NIH-3T3 cells with serial dilutions of the supernatant and subjecting the cells to G418 selection. A titer >10^6 CFU/mL is an appropriate starting point.
5. To obtain high viral titers, select for high-titer-producing clones of packaging cells using G418.
6. Once appreciable titers of the virus are obtained, filter the supernatant through a 0.4-µm cellulose acetate or polysulfonic (low-protein-binding) filter and store at –80°C.
7. To infect 32D cells, add an appropriate amount of the viral stock and polybrene (8 µg/mL) to the culture medium and incubate for 6 h.
8. Transfer the cells to fresh medium and add 600 µg/mL G418 after 24 h.
9. Select clones that express high levels of the receptor using fluorescence-assisted cell sorting.
10. To obtain high-expressing clones, do sequential infections ~12 h apart.
11. To analyze ligand-induced proliferative responses, wash cells free of IL-3, resuspend in IL-3 free medium, and seed 5.0 × 10^4 cells/well in a 96-well plate. For the time course experiment, prepare four identical 96-well plates and add a fixed amount of ligand (e.g. 100ng/ml). Incubate the cells at 37°C and perform the MTT assay on one plate every 24 h for 4 d. For dose-response experiments, add serial dilutions of ligand and incubate the cells for 24 h at 37°C. Use IL-3 (1:1000 of medium conditioned by a producer cell line) as a positive control.
12. Proliferation may be determined using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, which determines mitochondrial activity in living cells *(69)*. Incubate the cells for 2 h at 37°C with MTT

before performing a colorimetric assay. Living cells transform the tetrazolium ring into dark blue formazan crystals, which can be quantified by reading the OD at 540–630 nm after lysis of the cells with acidic 2-propanol.

13. While performing these experiments, one extra plate is also set up to determine the initial viability of the cells. These values serve as reference and the values obtained in the experiment is plotted in comparison to these values.

4. Notes

1. To compare affinity of two ligands for the same receptor, a simple displacement assay can be used. The concentration of an unlabeled ligand required to compete out 50% of the bound radiolabeled ligand is determined by keeping the concentration of the labeled ligand constant, and increasing the concentrations of the unlabeled ligand (for an example, *see* **ref. 55**).

2. Experiments similar to those described in **Subheading 3.2.1.** can be performed with cells transiently expressing the receptor or by using a cell line endogenously expressing the receptor. Dilution of the antibodies is as recommended by the supplier. The composition of the stripping buffer and the protocol may vary depending on the membrane used, the manufacturer's protocol is recommended.

3. In **Subheading 3.2.2.**, specific phosphopeptides can be used either to pull down proteins from cell lysates *(18)* or to compete *(19)* with receptor's motifs that interact with cytoplasmic proteins in immunoprecipitates. Quality of DNA is a determinant of transfection efficiency. In our laboratory, we commonly use Midi- and Maxi-Prep kits from Qiagen; kits from other companies may also be used.

4. In **Subheading 3.3.1.**, it is essential to determine the extent of nonspecific binding of EGF by using excess cold ligand. Other methods to follow endocytosis of ligand-receptor complexes are receptor recycling and ligand degradation assays *(70,71)*. An alternative nonradioactive assay in which the receptors are initially biotinylated using a cleavable form of biotin has also been reported *(72)*.

5. In **Subheading 3.3.2.**, an alternative method for the in vitro ubiquitination reaction is to use purified E1, E2, and E3 (Cbl) proteins expressed in *E. coli*.

6. Regulations for the use of retroviruses must be strictly followed and be performed by experienced personnel or under their supervision.

Acknowledgments

References

1. van der Geer, P., Hunter, T., and Lindberg, R. A. (1994) Receptor protein-tyrosine kinases and their signal transduction pathways. *Ann. Rev. Cell Biol.* **10,** 251–337.

2. Yarden, Y. and Ullrich, A. (1988) Growth factor receptor tyrosine kinases. *Ann. Rev. Biochem.* **57,** 443–478.

3. Yarden, Y. and Schlessinger, J. (1987) Epidermal growth factor induces rapid, reversible aggregation of purified epidermal growth factor receptors. *Biochemistry* **26,** 1443–1445.

4. Ullrich, A. and Schlessinger, J. (1990) Signal transduction by receptors with tyrosine kinase activity. *Cell* **61,** 203–212.

5. Blume-Jensen, P. and Hunter, T. (2001) Oncogenic kinase signaling. *Nature* **411,** 355–365.

6. Yarden, Y. and Sliwkowski, M. X. (2001) Untangling the ErbB signalling network. *Nat. Rev. Mol. Cell Biol.* **2,** 127–137.

7. Downward, J., Parker, P., and Waterfield, M. D. (1984) Autophosphorylation sites on the epidermal growth factor receptor. *Nature* **311,** 483–485.

8. Guy, P. M., Platko, J. V., Cantley, L. C., Cerione, R. A., and Carraway , K. L. (1994) Insect cell-expressed p180ErbB3 possesses an impaired tyrosine kinase activity. *Proc. Natl. Acad. Sci. USA* **91,** 8132–8136.

9. Klapper, L. N., Glathe, S., Vaisman, N., et al. (1999) The ErbB-2/HER2 oncoprotein of human carcinomas may function solely as a shared coreceptor for multiple stroma-derived growth factors. *Proc. Natl. Acad. Sci. USA* **96,** 4995–5000.

10. Tzahar, E., Waterman, H., Chen, X., et al. (1996) A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. *Mol. Cell. Biol.* **16,** 5276–5287.

11. Massague, J. and Pandiella, A. (1993) Membrane-anchored growth factors. *Ann. Rev. Biochem.* **62,** 515–541.

12. Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C., and Ullrich, A. (1999) EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* **402,** 884–888.

13. Beerli, R. R. and Hynes, N. E. (1996) Epidermal growth factor-related peptides activate distinct subsets of ErbB receptors and differ in their biological activities. *J. Biol. Chem.* **271,** 6071–6076.

14. Riese, D. J., Bermingham, Y., van Raaij, T. M., Buckley, S., Plowman, G. D., and Stern, D. F. (1996) Betacellulin activates the epidermal growth factor receptor and erbB-4, and induces cellular response patterns distinct from those stimulated by epidermal growth factor or neuregulin-beta. *Oncogene* **12,** 345–353.

15. Harari, D., Tzahar, E., Romano, J., et al. (1999) Neuregulin-4: a novel growth factor that acts through the ErbB-4 receptor tyrosine kinase. *Oncogene* **18,** 2681–2689.

16. Shelly, M., Pinkas-Kramarski, R., Guarino, B. C., et al. (1998) Epiregulin is a potent pan-ErbB ligand that preferentially activates heterodimeric receptor complexes. *J. Biol. Chem.* **273,** 10,496–10,505.

17. Olayioye, M. A., Graus Porta, D., Beerli, R. R., Rohrer, J., Gay, B., and Hynes, N. E. (1998) ErbB-1 and ErbB-2 acquire distinct signaling properties dependent upon their dimerization partner. *Mol. Cell. Biol.* **18,** 5042–5051.

18. Songyang, Z., Shoelson, S. E., Chaudhuri, M., et al. (1993) SH-2 domains recognize specific phosphopeptide sequences. *Cell* **72,** 767–778.

19. Batzer, A. G., Rotin, D., Urena, J. M., Skolnik, E. Y., and Schlessinger, J. (1994) Hierarchy of binding sites for Grb2 and Shc on the epidermal growth factor receptor. *Mol. Cell Biol.* **14,** 5192–5201.

20. Chang, L. and Karin, M. (2001) Mammalian MAP kinase signalling cascades. *Nature* **410,** 37–40.

21. Alroy, I. and Yarden, Y. (1997) The ErbB signaling network in embryogenesis and oncogenesis: signal diversification through combinatorial ligand-receptor interactions. *FEBS Lett.* **410,** 83–86.

22. Fedi, P., Pierce, J., Di Fiore, P. P., and Kraus, M. H. (1994) Efficient coupling with phosphatidylinositol 3-kinase, but not phospholipase Cγ or GTPase-activating protein, distinguishes ErbB-3 signaling from that of other ErbB/EGFR family members. *Mol. Cell Biol.* **14,** 492–500.

23. Shpakov, A. O. and Pertseva, M. N. (2000) Structural and functional characterization of insulin receptor substrate proteins and the molecular mechanisms of their interaction with insulin superfamily tyrosine kinase receptors and effector proteins. *Membr. Cell Biol.* **13,** 455–484.

24. Kouhara, H., Hadari, Y. R., Spivak-Kroizman, T., et al. (1997) A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. *Cell* **89,** 693–702.

25. Weidner, K. M., Di Cesare, S., Sachs, M., Brinkmann, V., Behrens, J., and Birchmeier, W. (1996) Interactions between Gab1 and c-Met receptor tyrosine kinase is responsible for epithel morphogenesis. *Nature* **384,** 173–176.

26. Hunter, T. and Karin, M. (1992) The regulation of transcription by phosphorylation. *Cell* **70,** 375–387.

27. Leevers, S. J. (1999) What goes up must come down. *Nat. Cell Biol.* **1,** E10, E11.

28. Vanhaesebroeck, B. and Alessi, D. R. (2000) The PI3K-PDK1 connection: more than just a road to PKB. *Biochem. J.* **346 (Pt. 3),** 561–576.

29. Datta, S. R., Brunet, A., and Greenberg, M. E. (1999) Cellular survival: a play in three Akts. *Genes Dev.* **13,** 2905–2927.

30. Cantley, L. C. and Neel, B. G. (1999) New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proc. Natl. Acad. Sci. USA* **96,** 4240–4245.

31. Hadari, Y. R., Kouhara, H., Lax, I., and Schlessinger, J. (1998) Binding of Shp2 tyrosine phosphatase to FRS2 is essential for fibroblast growth factor-induced PC12 cell differentiation. *Mol. Cell. Biol.* **18,** 3966–3973.

32. Penta, K. and Carpenter, G. (1997) Interaction of phospholipase C-gamma with activated growth factor receptor tyrosine kinases. *Adv. Exp. Med. Biol.* **400B,** 971–981.

33. Wells, A., Ware, M. F., Allen, F. D., and Lauffenburger, D. A. (1999) Shaping up for shipping out: PLCgamma signaling of morphology changes in EGF-stimulated fibroblast migration. *Cell Motil. Cytoskeleton.* **44,** 227–233.

34. Bao, J., Alroy, I., Waterman, H., et al. (2000) Threonine phosphorylation diverts internalized epidermal growth factor receptors from a degradative pathway to the recycling endosome. *J. Biol. Chem.* **275,** 26,178–26,186.

35. Simon, M. A. (2000) Receptor tyrosine kinases: specific outcomes from general signals. *Cell* **103,** 13–15.

36. Tan, P. B. and Kim, S. K. (1999) Signaling specificity: the RTK/RAS/MAP kinase pathway in metazoans. *Trends Genet.* **15,** 145–149.

37. Freeman, M. (1997) Cell determination strategies in the Drosophila eye. *Development* **124,** 261–270.

38. Schweitzer, R. and Shilo, B. Z. (1997) A thousand and one roles for the Drosophila EGF receptor. *Trends Genet.* **13,** 191–196.

39. Moghal, N. and Sternberg, P. W. (1999) Multiple positive and negative regulators of signaling by the EGF- receptor. *Curr. Opin. Cell Biol.* **11,** 190–196.

40. Sorkin, A., ed. (1996) *Receptor-Mediated Endocytosis of Growth Factors*, Chapman & Hall, London.

41. Hicke, L. (2001) Protein regulation by monoubiquitin. *Nat. Rev. Mol. Cell Biol.* **2,** 195–201.

42. Nesterov, A., Wiley, H. S., and Gill, G. N. (1995) Ligand-induced endocytosis of epidermal growth factor receptors that are defective in binding adaptor proteins. *Proc. Natl. Acad. Sci. USA* **92,** 8719–8723.

43. Santolini, E., Salcini, A. E., Kay, B. K., Yamabhai, M., and Di Fiore, P. P. (1999) The EH network. *Exp. Cell Res.* **253,** 186–209.

44. Levkowitz, G., Waterman, H., Ettenberg, S. A., et al. (1999) Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. *Mol. Cell.* **4,** 1029–1040.

45. Levkowitz, G., Waterman, H., Zamir, E., et al. (1998) c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. *Genes Dev.* **12,** 3663–3674.

46. Thien, B. F. and Langdon, W. Y. (2001) Cbl-Many adaptations to regulate protein tyrosine kinases. *Nature Rev. Mol. Cell Biol.* **2,** 294–305.

47. Jones, J. T., Akita, R. W., and Sliwkowski, M. X. (1999) Binding specificities and affinities of egf domains for ErbB receptors. *FEBS Lett.* **447,** 227–231.

48. Horan, T., Wen, J., Arakawa, T., et al. (1995) Binding of Neu differentiation factor with the extracellular domain of Her2 and Her3. *J. Biol. Chem.* **270,** 24,604–24,608.

49. Tzahar, E., Pinkas Kramarski, R., Moyer, J. D., et al. (1997) Bivalence of EGF-like ligands drives the ErbB signaling network. *EMBO J.* **16,** 4938–4950.

50. Lemmon, M. A., Bu, Z., Ladbury, J. E., et al. (1997) Two EGF molecules contribute additively to stabilization of the EGFR dimer. *EMBO J.* **16,** 281–294.

51. Garrett, T. P., McKern, N. M., Lou, M., et al. (1998) Crystal structure of the first three domains of the type-1 insulin-like growth factor receptor. *Nature* **394,** 395–399.

52. Pellegrini, L., Burke, D. F., von Delft, F., Mulloy, B., and Blundell, T. L. (2000) Crystal structure of fibroblast growth factor receptor ectodomain bound to ligand and heparin. *Nature* **407,** 1029–1034.

53. Plotnikov, A. N., Schlessinger, J., Hubbard, S. R., and Mohammadi, M. (1999) Structural basis for FGF receptor dimerization and activation. *Cell* **98,** 641–650.

54. Wiesmann, C., Fuh, G., Christinger, H. W., et al. (1997) Crystal structure at 1.7 A resolution of VEGF in complex with domain 2 of the Flt-1 receptor. *Cell* **91,** 695–704.

55. Shelly, M., Pinkas-Kramarski, R., Guarino, B. C., et al. (1998) Epiregulin is a potent pan-ErbB ligand that preferentially activates heterodimeric receptor complexes. *J. Biol. Chem.* **273,** 10,496–10,505.

56. Yung, Y., Dolginov, Y., Yao, Z., et al. (1997) Detection of ERK activation by a novel monoclonal antibody. *FEBS J.* **408,** 292–296.

57. Sorkin, A., Helin, K., Waters, C. M., Carpenter, G., and Beguinot, L. (1992) Multiple autophosphorylation sites of the epidermal growth factor are essential for receptor kinase activity and internalization. Contrasting significance of tyrosine 992 in the native and truncated receptors. *J. Biol. Chem.* **267,** 8672–8678.

58. Jeffers, M., Taylor, G. A., Weidner, K. M., Omura, S., and Vande Woude, G. F. (1997) Degradation of the Met tyrosine kinase receptor by the ubiquitin-proteasome pathway. *Mol. Cell. Biol.* **17,** 799–808.

59. Brock, R., Hamelers, I. H., and Jovin, T. M. (1999) Comparison of fixation protocols for adherent cultured cells applied to a GFP fusion protein of the epidermal growth factor receptor. *Cytometry* **35,** 353–362.

60. Carter, R. E. and Sorkin, A. (1998) Endocytosis of functional epidermal growth factor receptor-green fluorescent protein chimera. *J. Biol. Chem.* **273,** 35,000–35,007.

61. Sorkin, A., McClure, M., Huang, F., and Carter, R. (2000) Interaction of EGF receptor and grb2 in living cells visualized by fluorescence resonance energy transfer (FRET) microscopy. *Curr. Biol.* **10,** 1395–1388.

62. Deyholos, M. K. and Galbraith, D. W. (2001) High-density microarrays for gene expression analysis. *Cytometry* **43,** 229–238.

63. Hughes, T. R. and Shoemaker, D. D. (2001) DNA microarrays for expression profiling. *Curr. Opin. Chem. Biol.* **5,** 21–25.

64. Medico, E., Gambarotta, G., Gentile, A., Comoglio, P. M., and Soriano, P. (2001) A gene trap vector system for identifying transcriptionally responsive genes. *Nat. Biotechnol.* **19,** 579–582.

65. Hill, C. S. and Treisman, R. (1995) Transcriptional regulation by extracellular signals: mechanism and specificity. *Cell* **80,** 199–211.

66. Pierce, J. H., Ruggiero, M., Fleming, T. P., et al. (1988) Signal transduction through the EGF receptor transfected in IL-3-dependent hematopoietic cells. *Science* **239,** 628–631.

67. Pinkas-Kramarski, R., Soussan, L., Waterman, H., et al. (1996) Diversification of Neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. *EMBO J.* **15,** 2452–2467.

68. Riese, D. J., 2nd, van Raaij, T. M., Plowman, G. D., Andrews, G. C., and Stern, D. F. (1995) The cellular response to neuregulins is governed by complex interactions of the erbB receptor family. *Mol. Cell. Biol.* **15,** 5770–5776.

69. Mosman, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65,** 55–63.

70. Lenferink, A. E., Pinkas Kramarski, R., van de Poll, M. L., et al. (1998) Differential endocytic routing of homo- and hetero-dimeric ErbB tyrosine kinases confers signaling superiority to receptor heterodimers. *EMBO J.* **17,** 3385–3397.

71. Waterman, H., Sabanai, I., Geiger, B., and Yarden, Y. (1998) Alternative intracellular routing of ErbB receptors may determine signaling potency. *J. Biol. Chem.* **273,** 13,819–13,827.

72. Burke, P., Schooler, K., and Wiley, H. S. (2001) Regulation of epidermal growth factor receptor signaling by endocytosis and intracellular trafficking. *Mol. Biol. Cell.* **12,** 1897–1910.

determining their effects on MAPK enzymatic activities and on the transcriptional activity of transcription factors that act as downstream targets for these kinases. These represent basic tools for investigating the connection between GPCRs and MAPKs.

2. Materials

2.1. Cell Culture and Transfection

1. COS-7 and HEK-293 (referred to as 293) cells are maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (Life Technologies) and antibiotics (100 U/mL of penicillin G and 100 µg/mL of streptomycin; Life Technologies).
2. Solution of trypsin (0.05%) and ethylenediamine tetraacetic acid (EDTA) (0.53 mM; Life Technologies).
3. DEAE-dextran (Pharmacia LKB Biotechnology, Piscataway, NJ): 10 mg/mL stock solution in phosphate-buffered saline (PBS). Keep frozen.
4. Chloroquine (Sigma, St. Louis, MO): 100 mM in PBS. Keep frozen.
5. PBS.

2.2. Cell Stimulation and Lysis

1. The appropriate agonists for the receptors to be studied.
2. Lysis buffer: 25 mM HEPES, pH 7.5, 0.3 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT), 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 20 µg/mL aprotinin, 20 µg/mL leupeptin.

assaying the activation of the endogenous components of a given cell type and by analyzing the activity of ectopically expressed components, for those situations in which the activation of the endogenous system cannot be addressed directly. These basic protocols can be applicable to essentially all GPCRs and most cell types. In addition, the recent development of antibodies highly selective for the dual-phosphorylated, active forms of MAPKs has now afforded the possibility of examining the state of activation of these MAPKs by conventional techniques such as immunocytochemistry or Western blot analysis, and these techniques are also discussed.

2. Materials

All procedures involving cell cultures should be performed under sterile conditions. Solutions should be prepared in double-distilled MilliQ water. Radioactive materials should be handled with caution following standard laboratory safety procedures for protection and disposal of waste.

1. Cell culture media and plasticware.
2. Lysis buffer: 20 mM HEPES (pH 7.5), 10 mM EGTA, 40 mM β-glycerophosphate, 1% Nonidet P-40, 2.5 mM MgCl$_2$, 1 mM dithiothreitol, 2 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 µg/mL of aprotinin, 20 µg/mL of leupeptin. Store at 4ºC.
3. Wash buffer 1: phosphate-buffered saline (PBS) supplemented with 1% Nonidet P-40 and 2 mM sodium vanadate. Store at 4°C.
4. Wash buffer 2: 0.5 M LiCl in 100 mM Tris-HCl, pH 7.5. Store at 4°C.
5. Kinase reaction buffer: 10 mM 3-N Morpholino propane sulfonic acid (MOPS) (pH 7.5), 12.5 mM β-glycerophosphate, 7.5 mM MgCl$_2$, 0.5 mM EGTA, 0.5 mM sodium fluoride, 0.5 mM sodium vanadate. Store at 4°C.
6. [γ^{32}P]ATP (3000 Ci/mmol).
7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (5X): 50 mM Tris-HCl (pH 6.8), 10% SDS, 1 µg/mL of bromophenol blue, 50% glycerol.

3. Methods

3.1. Assaying Activation of MAPKs by GPCRs in a Physiologic Setting: ERK2 Activation by β-Adrenergic Receptors

In vitro MAPK assays are based on the ability of these proteins to act as phosphotransferase enzymes between a labeled donor substrate, [γ-^{32}P]ATP, and a protein acting as acceptor substrate (the kinase-specific substrate). Although the source of enzyme can be as different as crude cell lysate, cellular fractions, immunoprecipitates, partially purified proteins or purified enzymes, this section specifically deals with in vitro kinase assays performed on endogenous or transfected, epitope-tagged MAPKs isolated by immunoprecipitation with specific antibodies.

Many potent mitogens such as thrombin, lysophosphatidic acid (LPA), bombesin, vasopressin, bradykinin, substance K, acetylcholine receptor agonists, and angiotensin II stimulate cell proliferation by acting on their cognate GPCRs in a variety of cell types. Determining whether these mitogenic effects are associated with the activation of MAPKs has been a major goal during these last ten years *(1)*. The ideal situation to study the activation of MAPKs by GPCRs would be in a cellular setting in which the MAPKs and the GPCR under study are naturally expressed. This avoids subjecting cells to transfection reagents and the overexpression of exogenous DNAs.

Today the vast and ever growing number of cell lines in the different repositories throughout the world enables us, in most cases, to find an ideal cell line in which our GPCR of interest is expressed. Ideally, an assessment of the receptor numbers expressed in the cell type of our choice would be very useful. Otherwise, it is always a wise precaution to assay the expression of the GPCR in question by Western blot, when suitable antibodies are available.

Most, if not all, biochemical processes triggered by the stimulation of GPCRs follow a time-dependent course of activation that includes an early, sharp increase in the activation levels that eventually reach a maximum, after which the receptor desensitization takes place and the degree of activation gradually drops to resting levels. Thus, an essential parameter that must be determined when investigating the activation of MAPKs by GPCRs is the time course of the activation process, to pinpoint the period of stimulation after which maximum MAPK activation takes place. The optimal ligand/agonist concentration required to elicit maximum MAPK activation must also be determined. An agonist concentration below optimum levels may lead us to underestimate the activating potential of the GPCR under evaluation, while excess concentration may result in nonspecific effects and, at least, in an unnecessary waste of often very expensive agonists. Finally, the specificity of the response elicited by an agonist should be verified by the use of an appropriate antagonist, acting on the same receptor type. Pretreatment with the antagonist should prevent agonist binding to its cognate receptors, thus blocking the expected MAPK activation.

As an example of the aforementioned case, we describe the protocols followed to investigate the activation of ERK2 by β-adrenergic receptors that are naturally expressed in COS-7 cells *(6)*.

1. COS-7 cells are regularly grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Once confluent, starve the cells by culturing them overnight in serum-free medium (*see* **Note 1**).
2. Stimulate the cells with the β-adrenergic receptor agonist isoproterenol, at an estimated excess concentration such as 1 m*M*, for 1, 3, 5, 10, 20, 60, and 120 min.
3. At every time point, wash the cells in cold PBS.

4. Immediately lyse the cells in ice-cold lysis buffer, detach the cells with the aid of a cell scraper, and transfer to a 1.5-mL microcentrifuge tube.

5. Centrifuge at 15,000g for 20 min at 4ºC. Save the supernatants and transfer to clean tubes. Always keep the tubes on ice.

6. Measure the protein concentration by Bradford or other available methods. Always save 50–100 µg to monitor protein levels by Western blotting, if necessary.

7. Adjust the lysates from the different samples to the same protein contents (*see* **Note 2**), and perform the immunoprecipitation of ERK2 by adding 1 to 2 µg of a suitable anti-ERK2 antibody that retains kinase activity on immunoprecipitation (e.g., Santa Cruz SC-154; Santa Cruz Biotechnology) (*see* **Note 3**). Incubate on ice for 1 to 2 h.

8. Recover the immunocomplexes by adding 10 µL of protein G-Sepharose beads (Pharmacia Gamma-bind is recommended; Pharmacia) and keep at 4ºC for 15 min under shaking (*see* **Note 4**).

9. Give a pulse of centrifugation to pellet the beads.

10. Wash the pellet three times with wash buffer 1.

11. Wash the pellet once with wash buffer 2.

12. Wash the pellet once with kinase reaction buffer, and carefully aspirate all the remaining liquid.

13. Perform the kinase reaction in a total volume of 30 µL of kinase reaction buffer including the appropriate substrate, 1.5 mg/mL of myelin basic protein (MBP) in the case of ERK2 (*see* **Note 5**), 1.5 µL of 1 mM ATP, and 1 µL of [γ-^{32}P]ATP.

14. Incubate the reactions at 30°C for 20 min.

15. Stop the reactions by adding 10 µL of 5X loading buffer.

16. Boil the samples for 2 min, and spin and load in a 12% SDS-PAGE gel.

17. Once the electrophoresis has been performed, place the gel on Whatmann 3MM paper and dry at 80ºC under vacuum.

18. Expose the gel to autoradiography and/or quantitate using a PhosphorImager.

This protocol has been utilized to determine the course of activation of ERK2 on stimulation of β-adrenergic receptor by the agonist isoproterenol *(6)*. As shown in **Fig. 1**, ERK2 activation reaches a peak after 5 min of stimulation, decreasing thereafter, dropping to basal levels after 60 min. The specificity of the response is verified by the use of the β-adrenergic antagonist propanolol. Pretreatment with 20 µM propanolol for 20 min prior to the addition of isoproterenol completely precludes ERK2 activation by isoproterenol, but not by epidermal growth factor (EGF) (**Fig. 1**). Once the stimulation time course has been determined, one should proceed to investigate the optimal agonist concentration. For that purpose, the same experimental steps should be taken for cells stimulated with different concentrations of the agonist. As further examples, this method has been successfully utilized in our laboratories to study the activation of ERK2 induced in leukemic cells by differentiating agents, many of which act through GPCRs *(7,8)*, and to study the activation of JNK in trigeminal ganglia on stimulation with stress-inducing agents *(9)*.
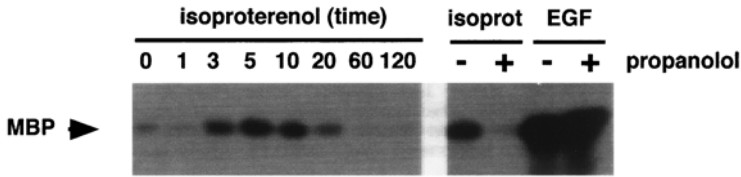
Fig. 1. MAPK activation by β-adrenergic receptors in COS-7 cells. Time course activation of MAPK by the ligand isoproterenol and its blockade by the antagonist propanolol is shown. Serum-starved cells were treated for the indicated times with 10 m*M* isoproterenol, and control cells or cells pretreated with 4 μ*M* propanolol for 20 min were stimulated for 5 min with isoproterenol or EGF (100 ng/mL). MAPK activation was determined in anti-ERK2 immunoprecipitates using MBP as a substrate.

3.2. Assaying Activation of MAPKs by GPCRs and Heterotrimeric G Proteins by Ectopic Expression of MAPKs

As a general rule, the protocol in **Subheading 3.1.** can be utilized to assay the activation of MAPKs in most, if not all, cellular settings. However, under some circumstances it may not be possible, or desirable, to analyze endogenous MAPKs activation. In these cases, it would be necessary to determine MAPK activity mirrored in a transfected, ectopic MAPK instead of the endogenous one. This is required, e.g., whenever a putative activating stimulus originates from an ectopically expressed protein. In these cases, the use of a cotransfected MAPK will enable the determination of MAPK activation only in those cells that have been successfully transfected and thus, have also incorporated the plasmids encoding for the stimulating protein. This becomes essential, e.g., whenever one wants to determine whether the activation of a given MAPK by a GPCR takes place through the Gα or the Gβγ components of the heterotrimeric G protein coupled to such a receptor. Because it is not possible to activate endogenous Gα or Gβγ components selectively and specifically, one must resort to their activation by overexpression, in the case of the βγ dimers, or in the case of the α-subunits, to the expression of activated mutants (QL mutants), whose intrinsic GTPase activity has been impaired, and thus are always in a GTP-bound, constitutively activated form.

To assay selectively the activation of the transfected MAPK, thus discriminating it from the endogenous MAPK activation, the ectopically expressed MAPK must be tagged. This is achieved by the addition of a small peptide sequence in frame with the coding sequence for each MAPK. There are at least a dozen of such epitopes described in the literature, and many commercially available mammalian expression vectors harbor these epitopes to enable easy

tagging of the protein of interest. We have successfully utilized MAPKs tagged with HA, Myc, AU5, and FLAG N-terminal epitopes (*see* **Note 6**). Whenever these epitopes are used, the corresponding antibody must be utilized in **Subheading 3.1.,** instead of the one raised against the endogenous MAPK.

Ectopic expression must also be utilized in those cases in which the nature of the molecules by which GPCRs activate MAPKs must be investigated. For that purpose, it will be necessary for the cotransfection of mammalian expression vectors encoding for the GPCR and MAPK of interest in addition to, e.g., a dominant inhibitory mutant of a given putative intermediary, in order to test its capability to block activation of the MAPK by the GPCR under investigation on addition of the corresponding agonist. Regarding the cellular setting in which these experiments are performed, ideally the cell of choice should be of physiologic relevance to those in which the GPCRs are naturally expressed. However, in this case transfectability is adamant, because a low transfection efficiency precludes many cell lines from being suitable for this type of experiment. Alternatively, cellular systems that provide high protein expression levels when utilizing vectors that carry the SV40 origin of replication, such as COS-7 or HEK 293T cells, can be handy substitutes.

3.3 Analysis by Antiphosphospecific Antibodies

Although grouped in at least four different subfamilies (ERKs, JNKs, p38s, and ERK5), all MAPKs require the contemporary phosphorylation of a tyrosine and threonine residue within their activation loop to be enzymatically active. Specific phosphorylation of these residues is usually performed by MAPK kinases, also known as MEKs *(10)*. To date, phosphorylation of the Thr-X-Tyr motif is the best known mechanism of MAPK regulation, and the amount of the dual-phosphorylated form of these proteins is usually considered a good estimate of their enzymatic activity. The use of antiphospho-MAPK specific antibodies is therefore a valuable tool to monitor the activity of endogenous MAPKs in response to different GPCR ligands, especially under conditions in which the amount of cellular lysate is not enough to perform immunoprecipitations. In addition, these reagents eliminate the need for ^{32}P labeling. The success of the use of such reagents is dependent on the quality of the phosphospecific antisera or monoclonal antibodies (MAbs), which, owing to the efforts of several companies, has improved dramatically over the last few years. Examples of phosphospecific antibodies that, in our experience, have yeilded high levels of sensitivity and reproducibility are Phospho-p44/42 MAPK (Thr202/Tyr204) MAb, Phospho-p38 MAP Kinase (Thr180/Tyr182), and Phospho-SAPK/JNK (Thr183/Tyr185) polyclonal antibodies from New England Biolabs; Phospho-JNK (pTPpY) polyclonal antibody from Promega (Madison, WI); and Phospho-p38 and Phospho-ERK5 from QCB-Biosource.

Ideally, parallel membranes should be probed with antisera or MAbs reacting with both the phosphorylated and unphosphorylated species of these MAPKs, thus facilitating evaluation of the fractional increase in the levels of the activated forms of these kinases.

4. Notes

1. The length of the starvation period depends on the MAPK considered and can be empirically determined. In our experience, the best results are usually obtained by overnight (12 h) serum starvation for ERKs and ERK5 kinase assays, and 2 to 3 h of starvation for assays measuring the activity of JNK, p38α, p38γ, and p38δ. It is also important to remember that most of these kinases are very sensitive to stress conditions such as those caused by changes in temperature and/or pH, or even prolonged serum starvation. Finally, to control the proper technical execution of the kinase assays, it is important to include an internal positive control for the experiment, treating the cells with a stimulus that is expected to increase the activity of the considered kinase. Examples of commonly used positive controls are LPA (5–10 μ*M*) or serum (10%) for ERKs; anysomicin (10 μg/mL) or NaCl (300 m*M*) for JNK, p38α, p38γ, and p38δ; and H_2O_2 (200 μ*M*) for ERK5.

2. MAPKs are relatively stable enzymes in the presence of protease inhibitors such as PMSF, leupeptin, and aprotinin. Calcium chelators (EDTA, EGTA) can similarly help to reduce the activity of calcium-activated proteases. Equally important, their activating Tyr/Thr phosphorylation can be easily preserved for the duration of the assay by keeping them constantly at 4°C, in buffers containing common phosphatase inhibitors (*see* **Subheading 3.**). Nevertheless, it is strongly recommended that control Western blots be performed to ascertain that the tested MAPK protein levels are similar in all samples.

3. Antibodies against ERKs, SAPKs/JNKs, p38s and BMKs are now commercially available from many companies (Santa Cruz Biotechnology, Oncogene Science, Transduction, Calbiochem) The Santa Cruz Biotechnology range is particularly extensive and generally works very well for Western blot and immunoprecipitations retaining kinase activities.

4. To pipet small volumes of protein G-Sepharose beads precisely, it is convenient to cut the end of the pipet tip.

5. Selecting a substrate normally depends on the specific kinase under evaluation. Even if each MAPK has been usually described to phosphorylate a number of specific proteins, only a few of them are of practical use as substrates in in vitro kinase assays. For example, MBP serves as a very good substrate for different MAPKs such as ERKs, p38α (HOG1), and p38γ (ERK6). Bacterially purified glutathione *S*-transferase (GST)-tagged ATF2 is the substrate of choice for JNK, even if GST-c-Jun is an equally good alternative for this kinase. GST-ATF2 also works as a good substrate to assay p38α (HOG1) kinase activity. Bacterially purified GST-MEF2C is the best substrate to assay ERK5 kinase activity. Nevertheless, if it is not available, MBP has also been successfully used in in vitro ERK5 kinase assays. While abundant amounts of MBP can usually be purchased

from different companies (e.g., Sigma), bacterially purified GST-ATF2, GST-c-Jun, and GST-MEF2C can be easily prepared by the researcher if expression vectors for the different fusion proteins are available. Otherwise, ready-to-use stocks of some of these proteins (GST-ATF2 and GST-c-Jun) can be purchased from several companies (e.g., Santa Cruz Biotechnology and New England Biolabs).

6. As an alternative to epitope tagging, GST-MAPK fusions can also be utilized. This avoids the use of immunoprecipitating antibodies because the GST fusions can be directly recovered by the addition of glutathione-Sepharose beads.

References

1. Marinissen, M. J. and Gutkind, J. S. (2001) G-protein-coupled receptors and signaling networks:emerging paradigms. *Trends Pharmacol. Sci*. **22,** 368–376.
2. Gutkind, J. S. (1998) The pathways connecting G protein-coupled receptors to the nucleus through divergent mitogen-activated protein kinase cascades. *J. Biol. Chem*. **273,** 1839–1842.
3. Gutkind, J. S. (1998) Cell growth control by G protein-coupled receptors: from signal transduction to signal integration. *Oncogene* **17,** 1331–1342.
4. Crespo, P., Xu, N., Simonds, W. F., and Gutkind, J. S. (1994) Ras-dependent activation of MAP kinase pathway mediated by G-protein beta gamma subunits. *Nature* **369,** 418–420.
5. Marinissen, M. J., Chiariello, M., Pallante, M., and Gutkind, J. S. (1999) A network of mitogen-activated protein kinases links G protein-coupled receptors to the c-jun promoter: a role for c-Jun NH2-terminal kinase, p38s, and extracellular signal-regulated kinase 5. *Mol. Cell. Biol*. **19,** 4289–4301.
6. Crespo, P., Cachero, T. G., Xu, N., and Gutkind, J. S. (1995) Dual effect of beta-adrenergic receptors on mitogen-activated protein kinase: evidence for a beta gamma-dependent activation and a G alpha s-cAMP-mediated inhibition. *J. Biol. Chem*. **270,** 25,259–25,265.
7. Visconti, R., Gadina, M., Chiariello, M., et al. (2000) Importance of the MKK6/p38 pathway for interleukin-12-induced STAT4 serine phosphorylation and transcriptional activity. *Blood* **96,** 1844–1852.
8. Ajenjo, N., Aaronson, D. S., Ceballos, E., et al. (2000) Myeloid leukemia cell growth and differentiation are independent of mitogen-activated protein kinaseERK1/2 activation. *J. Biol. Chem*. **275,** 7189–7197.
9. Pena, E., Berciano, M. T., Fernandez, R., Crespo, P., and Lafarga, M. (2000) Stress-induced activation of c-Jun N-terminal kinase in sensory ganglion neurons: accumulation in nuclear domains enriched in splicing factors and distribution in perichromatin fibrils. *Exp. Cell Res*. **256,** 179–191.
10. Pearson, G., Robinson, F., Beers Gibson, T., et al. (2001) Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr. Rev*. **22,** 153–183.

15

Identification of MAPK Substrates by Expression Screening With Solid-Phase Phosphorylation

Rikiro Fukunaga and Tony Hunter

1. Introduction

The biologic activities of protein kinases result from phosphorylation of their substrate proteins. Phosphorylation of proteins causes changes in structure, stability, enzymatic activity, ability to interact with other molecules, or subcellular localization, leading to regulation of a wide variety of cellular processes. Indeed, identification of physiologic targets has been a high priority ever since the first protein kinase was purified, but the conventional approach of purifying substrate proteins by biochemical techniques is a laborious and time-consuming task and is especially difficult in the case of scarce proteins. Among the approaches developed to identify protein kinase substrates in a systematic manner are various techniques for determining consensus phosphorylation site sequences using oriented peptide libraries *(1–3)*; interaction screening for protein substrates by an overlay method *(4,5)*; the yeast two-hybrid system *(6,7)*; and, more recently, the use of protein kinases engineered to accept unnatural adenosine triphosphate (ATP) analogs *(8–10)*; or protein kinase arrays *(11)*.

In this chapter, we describe a general screening method for identifying protein kinase substrates, termed phosphorylation screening, which utilizes a λ phage cDNA expression library with solid-phase phosphorylation. When applied to extracellular signal-regulated kinase 1 (ERK1) mitogen-activated protein kinase (MAPK), this strategy resulted in the isolation of several cDNAs encoding both known and novel substrates for ERK1 *(12)*. This method has also successfully been used to identify substrates for ERK2 MAPK *(13)*, cyclin E/Cdk2 *(14)*, Akt/PKB *(15)*, and PAK1 *(16)*. The method could be generally applicable for direct identification of physiologic targets of various protein kinases, including other MAPKs. An analogous phosphorylation screening

method has been developed for the identification of protein-tyrosine kinase substrates using a λgt11 cDNA expression library *(17)*.

1.1. Rationale for Solid-Phase Phosphorylation Screening of a Phage Expression Library

A λgt11-like phage expression library is screened by in vitro, solid-phase phosphorylation with [γ-^{32}P]ATP using a soluble preparation of the protein kinase of interest. An outline of this screening procedure is as follows:

1. A cDNA library is prepared using λGEX5 phage vector, in which a cDNA is inserted downstream of the glutathione *S*-transferase (GST) coding region *(12,18)*.
2. Phage plaques of the cDNA library are formed on agar plates, and then GST-fused recombinant proteins expressed in plaques are transferred and immobilized onto nitrocellulose filters.
3. The plaque filters are incubated with a purified, active protein kinase in the presence of [γ-^{32}P]ATP to allow solid-phase phosphorylation to occur. The phosphorylated plaques are visualized by autoradiography.
4. The positive clones are characterized by cDNA sequencing, and in vitro and in vivo analyses to identify physiologic substrates for the protein kinase.

The rationale for the solid-phase phosphorylation screening is based on the fact that cellular proteins immobilized on a membrane filter can be phosphorylated by a soluble protein kinase with specificity similar to that obtained in conventional liquid-phase phosphorylation *(19,20)*. Phosphorylation screening has several advantages over other methods such as substrate purification, determination of consensus peptide sequence, and interaction screening. The major advantages are as follows:

1. Even scarce proteins can be detected if they are efficient substrates.
2. Unlike peptide library screening methods, naturally existing proteins can be directly identified.
3. Unlike interaction screening, substrates that do not form a stable complex with the protein kinase can be detected.
4. Phosphorylation screening identifies only substrate proteins, whereas interaction screening is likely to detect also other protein kinase interactors such as subunits or regulatory proteins.
5. Cloned cDNAs can be easily sequenced and, because they are GST-fusion proteins, can readily be used for protein production, and therefore antibody preparation.

There are, however, some potential disadvantages and practical problems in the application of this technique:

1. If the protein kinase of interest phosphorylates an endogenous protein(s) derived from *Escherichia coli* or λ phage, it may be difficult to distinguish positive

plaques from negatives owing to a high background. This problem, however, could be minimized if an appropriate affinity system in which the recombinant products are selectively retained on a membrane filter is available (e.g., a glutathione [GSH]-derivatized cellulose filter).

2. A significant amount (e.g., microgram scale) of purified protein kinase is required in an active and soluble form.

3. This screening may isolate not only physiologic targets but also nonphysiologic and enzymologically preferred substrates, especially in the case of a protein kinase that has relatively low substrate specificity.

4. Protein kinases or other proteins that autophosphorylate may score positive in the screen, but this problem is reduced by a preincubation with unlabeled ATP and also minimized if the cDNA inserts are not too long. Using randomly primed cDNAs of ~1 kb in size for the library preparation might minimize problems with autophosphorylating protein kinases (*see* **Subheading 3.1.3.**).

Prior to using the phosphorylation screening technique, these advantages and disadvantages must be fully considered to judge whether this approach is suitable for the protein kinase in question.

2. Materials

2.1. Construction of λGEX5 cDNA Library

2.1.1. Preparation of λGEX5 Vector Arms (see **Subheading 3.1.2.**)

1. λGEX5 DNA: The λGEX5 vector is available on request through the DNA Bank Laboratory at RIKEN Gene Bank, the Institute of Physical and Chemical Research (RIKEN), Tsukuba 305-0074, Japan; www.rtc.riken.go.jp/DNA/HTML/engsearch.html. The ID number for the λGEX5 vector is 1911.
2. SDG buffer containing 10 or 40% sucrose: 10 mM Tris-HCl (pH 8.0), 0.9 M NaCl, 5 mM EDTA, 10 or 40% (w/v) sucrose.
3. TE buffer: 10 mM Tris-HCl (pH 8.0 or 7.5), 1 mM EDTA.

2.1.2. Preparation of SfiI Adaptor-Ligated cDNA (see **Subheading 3.1.3.**)

1. 5'-phosphorylated oligonucleotides
   a. 12mer: 5'-pd(CCAGCACCTGCA)-3'.
   b. 9mer: 5'-pd(AGGTGCTGG)-3'.
2. 10X Ligase buffer: 0.5 M Tris-HCl (pH 7.5), 0.1 M MgCl$_2$, 0.1 M dithiothreitol (DTT), 10 mM ATP.
3. T4 DNA ligase, T4 polynucleotide kinase, and *Sfi*I restriction enzyme (NEB or equivalent).
4. DNA ligation kit (PanVera/Takara version 1).
5. In vitro packaging kit (Stratagene Gigapack Gold, or equivalent).

2.2. Phosphorylation Screening

2.2.1. Plating Out λGEX5 cDNA Library and Immobilization of Plaques onto Nitrocellulose Filters (see **Subheading 3.2.2.**)

1. *E. coli* BB4 strain (Stratagene).
2. TB medium: 1% bacto-tryptone, 0.5% NaCl.
3. SM: 50 m*M* Tris-HCl (pH 7.5), 100 m*M* NaCl, 10 m*M* MgSO$_4$, 0.01% gelatin.
4. NZCYM medium: 1% NZ amine, 0.1% casamino acids, 0.5% bacto-yeast extract, 1% NaCl, 10 m*M* MgSO$_4$. Adjust the pH to 7.5 with 1 *N* NaOH.
5. Agar plates (60–80 mL of 1.5% agar in NZCYM medium/150-mm plate).
6. Top agarose (0.7% agarose in NZCYM medium).
7. Nitrocellulose filters (137 mm in diameter, Schleicher & Schuell BA85 (0.45 μm) or equivalent).
8. Isopropyl β-D-thiogalactopyranoside (IPTG).

2.2.2. Solid-Phase Phosphorylation With ERK1 MAPK and Identification and Isolation of Positive Clones (see **Subheadings 3.2.3.** and **3.2.4.**)

1. Rotating platform.
2. 150-mm Tissue culture dishes (Falcon #3025, or equivalent).
3. Blocking solution: 20 m*M* Tris-HCl (pH 8.0), 150 m*M* NaCl, 3% bovine serum albumin (BSA) (Fraction V; Sigma), 1% Triton X-100.
4. Triton wash buffer: 20 m*M* Tris-HCl (pH 7.5), 150 m*M* NaCl, 10 m*M* EDTA, 1 m*M* EGTA, 0.5% Triton X-100, 1 m*M* DTT, 0.2 m*M* phenylmethylsulfonyl fluoride (PMSF).
5. MAPK reaction buffer: 20 m*M* HEPES-NaOH (pH 7.5), 10 m*M* MgCl$_2$, 50 μ*M* Na$_3$VO$_4$, 5 m*M* β-glycerophosphate, 5 m*M* NaF, 2 m*M* DTT, 0.1% Triton X-100.
6. MAPK wash buffer: 20 m*M* Tris-HCl (pH 7.5), 150 m*M* NaCl, 10 m*M* EDTA, 1 m*M* EGTA, 20 m*M* NaF, 0.1% Triton X-100.

2.3. Conversion of Phage Clones into Plasmids

2.3.1. Rapid Small-Scale Preparation of λGEX5 Phage DNA (see **Subheading 3.3.**)

1. λ diluent: (10 m*M* Tris-HCl, pH 7.5, 10 m*M* MgSO$_4$.
2. 50X DNase/RNase mixture (0.1 mg/mL of DNase I, 1 mg/mL of RNase A). Make up 1X DNase/RNase mixture in λ diluent just before use.
3. 35-mm Agarose plates in six-well tissue culture plate (3 mL of 1.5% agarose in NZCYM medium/well) (Note that agarose but not agar should be used, since phage DNA prepared from agar plates is often resistant to restriction enzyme digestion.)
4. Extraction buffer: 10 m*M* Tris-HCl (pH 8.0), 0.1 *M* NaCl, 10 m*M* EDTA, 0.1% sodium dodecyl sulfate (SDS).

2.3.2. Excision Rescue of cDNA-Containing Plasmid From λGEX5 Clones *(see **Subheading 3.3.**)*

1. *Not*I reaction mixture: for 10 reactions, 48 μL of water, 10 μL of 10X *Not*I buffer (NEB or equivalent), and 2 μL (20 U) of *Not*I. Prepare the mixture on ice just before use.
2. Ligase reaction mixture: for 10 reactions, 40 μL of water, 7 μL of 10X ligase buffer (*Protocol 2*), and 3 μL (300–1200 U) of T4 DNA ligase. Prepare the mixture on ice just before use.
3. XL1-Blue competent cells (Stratagene).
4. 2X YT medium: 1.6% bacto-tryptone, 1% bacto-yeast extract, 0.5% NaCl.

2.4. Initial Characterization of Candidate Clones

2.4.1. Analysis of GST-Fusion Proteins Produced by Candidate Clones *(see **Subheading 3.4.1.**)*

1. Sonicator with a microtip.
2. Lysozyme solution: 5 mg/mL in 0.1 *M* Tris-HCl (pH 8.0).
3. TLB: 50 m*M* HEPES-NaOH (pH 7.4), 150 m*M* NaCl, 10% glycerol, 1% Triton X-100, 1 m*M* EGTA, 1 m*M* DTT, 1 m*M* PMSF.
4. GSH-agarose beads (Sigma, or equivalent).
5. 2X SB: 0.1 *M* Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 40% 2-mercaptoethanol, 0.04% bromophenol blue.

3. Methods

3.1. Construction of λGEX5 cDNA Library

3.1.1. λGEX5 Vector

For this screening method, we constructed a phage vector, λGEX5 *(12)*, by modifying the λgt11 vector, which has been successfully used for various expression screening strategies such as immunoscreening, southwestern nucleic acid–protein interaction, and protein-protein-interaction (also called far-western) *(21,22)*. The λGEX5 vector contains a plasmid sequence inserted between the two *Not*I sites, which consists of the ColE1 origin, the ampicillin resistance gene, and the GST coding sequence followed by a small (0.43-kb) stuffer sequence (**Fig. 1**). The nucleotide sequence of the plasmid region (pGEX-PUC-3T; *see* **Fig. 4**) is available in the DDBJ/EMBL/GenBank database (accession no. AB014641). The *Sfi*I sites at the ends of the stuffer region are used for insertion of cDNAs of up to 9 kb in size, which can be expressed as GST-fusion proteins on induction by IPTG. The λGEX5 vector system has additional advantages over the original λgt11 vector:
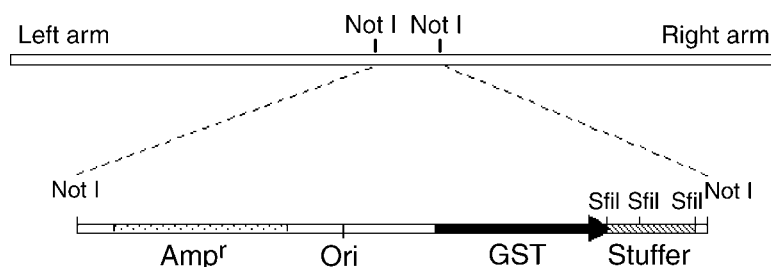
Fig. 1. Structure of λGEX5 cloning vector. The plasmid region between two *Not*I sites is shown in the lower part.

1. Isolated clones can be rapidly converted into plasmid clones by excision rescue without purifying cDNA fragments.
2. The rescued plasmids can be directly utilized not only for cDNA sequencing but also for expression of the GST-fusion proteins, which can be used for further characterization as substrates, and so on.
3. GST, the N-terminal fusion partner of the recombinant product expressed by λGEX5, is highly soluble, easy to purify by GSH-agarose chromatography, and much smaller (27 kDa) than the β-galactosidase (114-kDa) component of fusion proteins expressed by λgt11 *(22)*.

These advantages enable rapid isolation, subcloning, and characterization of a large number of positive clones at the same time.

3.1.2. Preparation of Vector Arms

λGEX5 phage DNA is purified from large quantities (10^{12}–10^{13} PFU) of phage lysate according to the established method *(23,24)*. The λGEX5 phage contains an amber mutation (*S*am100) and should be propagated using *E. coli* BB4 *(25)* as the host strain. Other strains such as Y1090 *(22)* and its derivatives may not be suitable. If it is hard to obtain a high-titer phage lysate by liquid culture, a large-scale plate lysate (e.g., from twenty 150-mm agarose plates) should be used instead *(23,24)*.

Vector arms for construction of cDNA library are prepared by *Sfi*I digestion of the λGEX5 DNA. Dephosphorylation of the cleaved sites is not necessary because the 3'-protruding, single-stranded termini (3'-CGT) of the arms are not compatible with each other. The existence of an additional *Sfi*I site in the stuffer sequence helps to check whether or not the *Sfi*I digestion is complete (**Fig. 2**). After confirming that the digestion is complete, the left arm (22.5 kb) and right arm (18.9 kb) are purified together by sucrose density gradient centrifugation or by preparative agarose gel electrophoresis *(23,24)*.

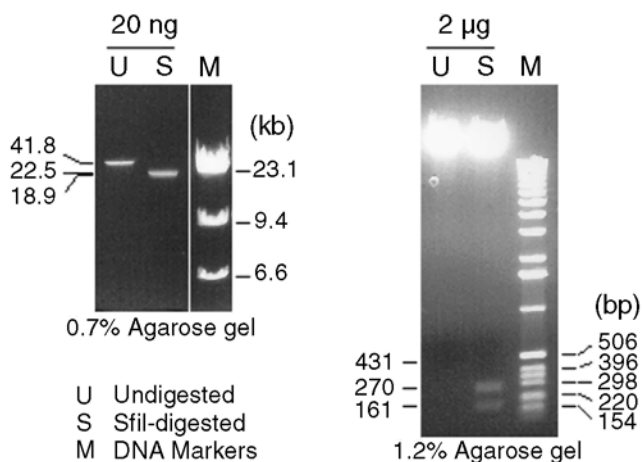1. Digest 80 μg of λGEX5 DNA with *Sfi*I restriction enzyme.

Fig. 2. Confirmation of complete *Sfi*I digestion of λGEX5 DNA for preparation of vector arms. Undigested or *Sfi*I-digested λGEX5 DNA was analyzed by electrophoresis on 0.7% **(left)** and 1.2% **(right)** agarose gels. The left arm (22.5 kb) and right arm (18.9 kb) are not clearly separated. Note that no DNA fragment is visible at the position of 431 bp.

2. Analyze two aliquots (20 ng and 2 μg) of the digested DNA by 0.7 and 1.2% agarose gel electrophoreses, respectively. It is important to confirm that no DNA is visible at the position of the undigested λGEX5 DNA on the 0.7% gel. On the 1.2% gel, 270- and 161-bp bands but not a 431-bp band should be visible. If the 431-bp band is detectable, it means that the *Sfi*I digestion is incomplete, which will cause a high ratio of empty phage clones in the library.

3. Extract the digested DNA once with phenol:chloroform and once with chloroform, and recover the DNA by ethanol precipitation.

4. Dissolve the DNA in 0.4 mL of TE (pH 8.0), add 4 μL of 1 *M* MgCl$_2$, and incubate for 1 h at 42°C to allow the cohesive termini of the arms to anneal.

5. Prepare two 12-mL 10–40% linear sucrose gradients in two centrifuge tubes (Beckman SW41 or equivalent).

6. Load 0.2 mL of the annealed DNA (40 μg) onto each gradient and centrifuge at 130,000*g* for 16 h at 15°C in a Beckman SW41.Ti rotor (or equivalent).

7. Collect 10 drops (0.6–0.7 mL) of fractions through a 20-gage needle from the bottom of the centrifuge tube.

8. Remove 10 μL of each fraction and analyze by 0.7% agarose gel electrophoresis.

9. Pool the fractions that contain the vector arms but not the stuffer fragments. Dilute the pooled fractions with an equal volume of TE (pH 7.5) and recover the DNA by ethanol precipitation.

3.1.3. Preparation of SfiI Adapter-Ligated cDNA

Although synthesis of double-stranded cDNA is an important step for cDNA cloning, a detailed description of this step is not the purpose of this chapter. Double-stranded cDNA can be synthesized from poly(A)$^+$RNA with oligo(dT) primer or random hexanucleotide primer by a general method for cDNA synthesis *(23,24)*, or by using an appropriate kit. Oligo(dT)-primed cDNA libraries are prone to be rich in clones that contain only the C-terminal part of a protein. On the other hand, randomly primed cDNA libraries evenly cover every part of a protein in principle, although they often contain a high percentage of cDNAs for ribosomal RNAs. At the final stage of cDNA synthesis, the ends of the double-stranded cDNA should be blunted for adapter ligation. Two 5'-phosphorylated oligonucleotides are annealed to form an adapter, which has a blunt end and a nonpalindromic, single-stranded overhang (3'-ACG) compatible with the cloning sites of the *Sfi*I-digested λGEX5 arms. After adaptor ligation, the cDNA is separated by agarose gel electrophoresis, and the cDNA whose size is larger than ~0.8 kb in length is recovered by electroelution. If the cDNA is synthesized with random primers, it may be a good idea to fractionate only cDNA of ~1 kb in size in order to minimize problems with autophosphorylating protein kinases. A large amount of excess oligonucleotides can be removed through the size fractionation step.

1. Make up an oligonucleotide mixture containing 2.4 and 1.8 μg of the 12mer and 9mer oligonucleotides, respectively, in 20 μL of 10 m*M* MgCl$_2$.
2. Incubate the mixture at 80°C for 2 min, allow it to cool slowly to room temperature over a period of about 60 min, and then chill on ice.
3. Mix in the following order:
   a. 55 μL of H$_2$O.
   b. 20 μL of the annealed oligonucleotides.
   c. 10 μL (1 to 2 μg) of double-stranded cDNA.
   d. 10 μL of 10X ligation buffer.
   e. 1 μL of 10 U/μL of T4 polynucleotide kinase.
   f. 4 μL of 400 U/μL of T4 DNA ligase.
4. Incubate the mixture (100 μL) for 6–14 h at 16°C. Recover the DNA by phenol:chloroform extraction and ethanol precipitation.
5. Dissolve the adapter-ligated cDNA in 20–50 μL of TE (pH 8.0), and separate the DNA fragments by preparative electrophoresis on a 1% agarose gel.
6. Locate the region of DNA fragments larger than 0.8 kb in size by using appropriate DNA size markers, and recover the size-fractionated DNA from the agarose gel by electroelution.

3.1.4. Ligation and In Vitro Packaging

The adapter-ligated and size-fractionated cDNA molecules can now be ligated to the *Sfi*I-digested vector arms, followed by packaging of the ligated

DNA into bacteriophage λ particles by an in vitro packaging reaction. We usually use commercially available kits for this ligation and in vitro packaging according to manufacturer's instructions. It is desirable to carry out pilot ligations to optimize the ratio of cDNA to the vector arms for efficient production of recombinant phages. In addition, a control ligation without cDNA is essential to check the background level of empty phage. The titer of the in vitro-packaged phage is determined on *E. coli* BB4. The plaque forming units in the original, packaged phage is the number of independent clones in the library. If necessary, the cDNA library can be amplified once by propagating phages in *E. coli* BB4 on agar plates *(23,24)*.

3.1.5. Construction of a Positive Control Phage for Screening

In using phosphorylation screening, conditions for screening must be determined experimentally for each protein kinase. For this purpose, ideally one needs to have a positive control phage that expresses the GST-fusion of an appropriate substrate protein (or peptide) for the protein kinase being used for the screening (assuming that one is known). The positive control phage can be constructed by ligating the *Sfi*I-digested λGEX5 arms to an appropriate substrate cDNA produced by polymerase chain reaction with *Sfi*I site-containing primers. For the screening of ERK1 MAPK substrates, we constructed a λGEX5 recombinant encoding the C-terminal region of a transcription factor Elk-1 (λGEX-Elk-C), which contains multiple ERK phosphorylation sites *(12,26)*. Similarly, a GST fusion of the C-terminal domain of the retinoblastoma protein (GST-Rb) worked well as a positive control in a screen for cyclin E/Cdk2 substrates *(14)*.

3.2. Phosphorylation Screening

In this section we describe protocols for solid-phase phosphorylation, using as an example substrate screening with ERK1 MAPK *(12)*. Points to be considered for application to other protein kinases are also discussed.

3.2.1. Preparation of Protein Kinase for Phosphorylation Screening

The protein kinase used for phosphorylation screening must be soluble, active and satisfactorily pure. Care should be taken to avoid the presence of significant amounts of other protein kinases. Large-scale production and purification of recombinant protein kinase would be the best for this purpose. To obtain a large amount of activated ERK1 MAPK, we produced recombinant human ERK1 using a baculovirus expression system, in which insect Sf9 cells were coinfected with three recombinant baculoviruses encoding v-Ras, c-Raf-1, and ERK1 as described *(12,27)*. Similar coexpression strategies utilizing a eukaryotic expression system would also be effective for large-scale produc-

tion of a protein kinase that requires a subunit protein or an activating modification such as phosphorylation. For example, active cyclin-Cdk complexes can be efficiently produced by coexpression of a hexahistidine-tagged cyclin and its partner Cdk in Sf9 cells, and easily purified using a nickel-affinity column chromatography *(14)*.

3.2.2. Preparation of Plaque-Immobilized Filters

Procedures in this step are essentially same as those used for expression screens using the λgt11 phage system *(21–24)*. A λGEX5 cDNA library is plated on agar plates, and expression of the encoded GST-fusion proteins is induced by overlaying IPTG-containing nitrocellulose filters onto the plaques. Plating density should be in a range of $1.5–3 \times 10^4$ plaques/150-mm agar plate. A high-density plating ($>5 \times 10^4$ plaques/plate) may result in weak and small-diameter signals that are indistinguishable from false-positive signals.

1. Pick up a single colony of BB4 and grow the cells in TB medium containing 0.2% maltose, 10 mM MgSO$_4$ and 12.5 μg/mL of tetracycline at 30°C overnight (since BB4 contains F' factor encoding *lacI*^{q} and *tet*^{r} genes, the original strain should be maintained in the presence of tetracycline). Centrifuge the cells at 1500g for 10 min and resuspend in 10 mM MgSO$_4$ at a density of OD$_{600}$ = 2.0. The plating bacteria can be stored at 4°C up to 1 wk.
2. Prepare $1.5–2 \times 10^5$ PFU/mL of λGEX5 library phage in SM.
3. Mix 0.1 mL (i.e., $1.5–2 \times 10^4$ PFU) of the phage with 0.5 mL of plating bacteria, and incubate for 15 min at 37°C.
4. Add 8 mL of molten (50°C) top agarose, mix well, and pour onto 1.5% agar plates prewarmed at 37°C. Leave the plates at room temperature for 15 min to harden the top agarose.
5. Incubate at 42°C for 3–4 h.
6. Soak nitrocellulose filters with 10 mM IPTG, and remove the excess liquid by laying on Whatman 3MM paper.
7. Overlay the plates with the IPTG-impregnated nitrocellulose filters. Do not allow the plates to cool.
8. Incubate at 37°C for another 6–10 h.

3.2.3. Solid-Phase Phosphorylation

The next step is incubation of the plaque-immobilized filters in a reaction buffer containing protein kinase and [γ-^{32}P]ATP. To avoid nonspecific adsorption of the purified protein kinase onto the nitrocellulose filters, the filters need to be blocked with a protein that does not bind to and is not phosphorylated by the protein kinase in question, and also lacks detectable protein kinase activity. For ERK1 MAPK and cyclin E/Cdk2, we found BSA to be a suitable blocking agent. Prior to the phosphorylation step, the filters are then incubated in the presence of unlabeled ATP. The purpose of this preincubation step is to reduce

the frequency of isolating clones whose products have an autophosphorylating or ATP-binding activity as discussed in **Subheading 1.1.** The following protocol describes an example of this step with conditions that were optimized to carry out substrate screening for ERK1 MAPK *(12)*.

1. Cool the plates to room temperature. Mark the filters asymmetrically with a needle.
2. Carefully peel off the filters and immerse one by one into a large volume (at least 200 mL for 20 filters) of blocking solution, which contains BSA to block remaining unoccupied protein binding sites on the filter. Throughout the following steps, filters should be kept wet and under gentle (or in some steps, relatively strong) agitation.
3. Agitate the filters slowly on a rotating platform for 60 min at room temperature.
4. Wash the filters three times for 20 min at room temperature in 200–300 mL of Triton wash buffer. Slightly stronger agitation may be required to remove bacterial debris from the filters.
5. Wash the filters for 10 min at room temperature in 200 mL of MAPK reaction buffer.
6. Incubate the filters for 60 min at room temperature in 200 mL of MAPK reaction buffer containing 25–100 μM unlabeled ATP to mask proteins that have autophosphorylating and/or ATP-binding activities.
7. Wash the filters for 10 min in 200 mL of MAPK reaction buffer without ATP.
8. Incubate the filters for 60 min at room temperature (or 30°C, if an air incubator is available) with gentle shaking in the MAPK Reaction Buffer containing 25 μM unlabeled ATP, 5 μCi/mL of [γ-^{32}P]ATP, and 1 μg/mL of purified human ERK1 MAPK. Use at least 2 mL of the solution/137-mm filter. A 150-mm tissue culture dish is convenient for the incubation of up to 20 filters.
9. Wash the filters six to seven times for 5–10 min at room temperature in 100–200 mL of MAPK wash buffer. Relatively strong agitation helps to reduce false-positive signals. Finally wash the filters once with MAPK wash buffer without Triton X-100.
10. Dry the filters on paper towels, and arrange on a paper sheet for autoradiography.

3.2.4. Identification of cDNA Clones Encoding Substrate Candidates

The phosphorylated filters are exposed to X-ray film to identify positive plaques by autoradiography. The intensities of radioactive signals are generally quite variable from plaque to plaque, presumably because the recombinant proteins are expressed at different levels, and also because they are not all equally efficient substrates and are therefore phosphorylated to different extents. Thus, for practical reasons, it is advisable to pick up only a limited number (e.g., ~100) of clones in order of intensity from the plates in the primary screening. It may often be difficult to discriminate truly phosphorylated plaques from false-positive spots. However, this problem can be overcome by making an additional autoradiograph of the same filter with much shorter exposure. Generally speaking, pinpoint signals with a sharp rim found in the short exposure are false positives, whereas true-positive plaques give rather

dull, fuzzy signals with a certain size range. Therefore, most false-positive signals can be easily excluded by carefully comparing signals at the corresponding position on the two autoradiographs. It is important, if possible, to include a positive λGEX-substrate control plate (*see* **Subheading 3.1.5.**), because this will indicate the sort of signal intensity and morphology to expect for true-positive plaques in the screen. Alternatively, primary screening may be carried out in duplicate *(24)*. By comparing the duplicate filters, true plaques can be easily identified as reproducible signals. Since most plaques are in contact with one another in the primary screening, phage clones must be purified by a secondary screening step in which phages are plated out sparsely. This step also serves to confirm whether or not the plaques identified in the primary screening are truly phosphorylated by the protein kinase.

1. Expose the phosphorylated filters to X-ray films for 4–72 h at –70°C with an intensifying screen. Make at least two sets of autoradiographs with different exposure times: one with a sufficiently long exposure and the other with a much shorter exposure.
2. Mark strong signals on the long-exposure films and carefully observe their corresponding signals on the short-exposure films. Identify sharp, pinpoint signals on the short-exposure films, which are likely to be false positives. Mark them again differentially on the long-exposure films for exclusion.
3. Align the long-exposure films with the filter sheets. Mark the positions of the asymmetric needle spots on the filters.
4. Identify the locations of positive plaques, and remove a relatively large agar plug (2–4 mm in diameter) from each position to be certain of recovering the positive clone.
5. Transfer the agar plug to 1 mL of SM containing two drops of chloroform, and incubate for 1–2 h at room temperature. Measure the titer of each phage stock.
6. Replate each phage stock onto a 90-mm agar plate at a low density (200–500 plaques/plate), and transfer the plaques to 85-mm nitrocellulose filters as described earlier in this section.
7. Repeat solid-phase phosphorylation of the plaque lift filters as described in **Subheading 3.2.3.** Use 0.7 mL of the kinase reaction buffer/filter. Expose the phosphorylated filters to X-ray films.
8. Identify a single, well-isolated positive plaque (clone) on each plate. If it is difficult to obtain a single plaque, repeat **steps 3–8** to purify the positive phage clone.

Figure 3 shows a typical result of solid-phase phosphorylation using ERK1 MAPK. In the control experiment with positive (λGEX-Elk-C) and negative (λGEX5) control phages, these two phages gave a clear contrast in intensity of radioactive signals. In the actual screening of an HeLa cDNA library, however, signals of various degrees of intensity were observed, as discussed above.

3.2.5. Optimization of Screening Condition Using Control Phages

Since optimum conditions of phosphotransfer reaction are quite variable among protein kinases, screening conditions should be experimentally deter-

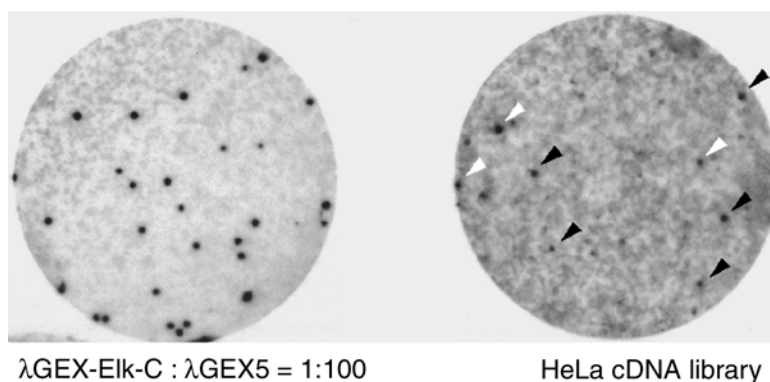λGEX-Elk-C : λGEX5 = 1:100   HeLa cDNA library

Fig. 3. Autoradiograph of solid-phase phosphorylation screen. Phage plaques (4000 PFU/90-mm agar plate) were transferred to 85-mm nitrocellulose filters and then subjected to phosphorylation screening with ERK1 MAPK according to **Subheadings 3.2.2.** and **3.2.3. (Left)** A 1:100 mixture of the positive control phage (λGEX-Elk-C) and a negative control phage (λGEX5). **(Right)** Screening of HeLa cDNA library. Black arrow ends indicate positive plaques whereas white arrow ends indicate false positives. Although these signals cannot be distinguished in this figure, signals indicated by open triangles showed up as pinpoint spots on a short-exposure film (not shown).

mined for each protein kinase being used. Components of the reaction buffer to be considered would be pH, species and concentrations of monovalent and divalent cations, type of detergent, protease inhibitors, phosphatase inhibitors, other additives, and so forth, depending on the enzymologic properties of the protein kinase. The presence of some detergent (e.g., Triton X-100, Nonidet P-40, Tween-20) in the buffers is helpful to reduce false-positive signals, most of which seem to be derived from tiny dust or bacterial debris. Thus, it is desirable to include a detergent at least in the washing buffers unless the protein kinase is highly sensitive to it. There are no general rules for the concentration and specific radioactivity of $[\gamma\text{-}^{32}P]ATP$, concentration of protein kinase, reaction temperature, and duration. Therefore, the first trial should be performed in the reaction mixture that is usually used for the in-solution kinase assay of the protein kinase. In practice, however, a simple scaling up from test tube (10–50 μL) to culture dish (10–50 mL) may not be easy, especially for the quantities of the protein kinase and radioactive ATP needed. Fortunately, reduction in concentration of these components may be compensated to some extent by raising the specific activity of $[\gamma\text{-}^{32}P]ATP$ (i.e., reducing the concentration of unlabeled ATP) and by changing the incubation time for phosphorylation and the exposure time of autoradiography. Optimization of these three parameters is particularly important to obtain a good signal-to-noise ratio in autoradiograph.

Ideally, determination of screening conditions should be carried out using positive and negative control phages. If a substrate for the protein kinase in question is already known, a positive control phage can be constructed as described in **Subheading 3.1.5.** If not, reaction conditions may need to be checked while an actual screen is being performed with a cDNA library, assuming that positive clones exist in it. The λGEX5 vector phage can usually be used as a negative control phage although this phage expresses a GST-fusion protein with an artificial C-terminal sequence derived from the stuffer region (GST-stuffer). If the stuffer-derived sequence (58 amino acids; DDBJ/ EMBL/GenBank accession no, AB014641) contains any potential phosphorylation sites for the protein kinase, it may be necessary to construct another control phage expressing only the GST region. It is desirable to check in advance whether the GST-fused substrate protein (or peptide) is phosphorylated by the protein kinase much more efficiently than the negative construct (GST-stuffer or GST itself) in a test tube assay (e.g., *see* Fig. 5, GST and GST-Elk-C lanes). If the fusion protein is not phosphorylated or is poorly phosphorylated, other constructs should be considered. Furthermore, there is a possibility that GST itself acts as a good substrate for the protein kinase. In this case, other vector systems such as λgt11 *(21,22)* or λZAP *(28)* may have to be used instead.

The control experiment can be performed using 90-mm agar plates on which either of the control phages and a series of mixtures of the two control phages are plated out at a density of $4–8 \times 10^3$ plaques/filter. The ratios of the positive and negative control phages in the mixtures should be, e.g., 1:10, 1:100, and 1:1000. The autoradiograph of solid-phase phosphorylation of these filters helps to estimate the difference in intensity between positive and negative signals, background radioactivity of plaques and the *E. coli* lawn, and frequency of appearance of false-positive signals. If the model experiment works well, the number of strong signals should change among the filters in direct proportion to the ratio of the positive phages in the mixtures (**Fig. 3**).

3.3. Conversion of Phage Clones into Plasmids

If positive plaques give clearly stronger signals than the remaining clones in the secondary screening, this means that the first step of the screening is successful. For further characterization, positive clones now have to be converted into plasmids that contain their cognate cDNA. The following protocol (**Subheading 3.3.1.**) describes a rapid plate lysate method for preparing DNA from multiple (10–100) phage clones at the same time. The cDNA-containing plasmid (pGEX-PUC-3T) can be recovered by *Not*I digestion of the phage DNA followed by self-circularization, as described in the next protocol (**Subheading 3.3.2.**). We recommend *E. coli* XL1-Blue *(25)* as the host strain for the
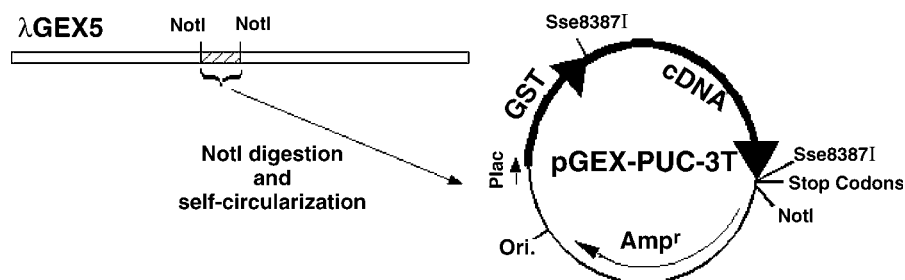
Fig. 4. Schematic representation of excision rescue. After digestion of phage DNA with *Not*I, the plasmid region containing cDNA can be recovered by self-ligation. In the resulting pGEX-PUC-3T plasmid, the *Sfi*I sites used for cDNA cloning are not regenerated, but *Sse*8387I sites are available to excise the cDNA fragment.

transformation of this plasmid. A schematic representation of this process is shown in **Fig. 4**.

3.3.1. Rapid Plate Lysate

1. Using a Pasteur pipet, pick a single plaque and place in 0.5 mL of SM containing one drop of chloroform. Incubate the suspension for 1 to 2 h at room temperature.
2. Mix 20 μL of the phage suspension with 30 μL of plating bacteria (BB4, $OD_{600} =$ 2.0) and incubate for 15 min at 37°C.
3. Add 0.4 mL of molten (50°C) top agarose (0.7% agarose in NZCYM medium), and spread the bacterial suspension on the surface of 35-mm agarose plates in six-well tissue culture plates.
4. Incubate at 37°C overnight (10–16 h) to reach confluent lysis.
5. Directly add 0.65 mL of λ diluent containing 2 μg/mL of DNase I and 20 μg/mL of RNase A onto the surface of the top agarose, and incubate for 1.5–2 h at room temperature with constant, gentle shaking.
6. Transfer the phage suspension (usually 0.4 to 0.5 mL) to a microfuge tube, add 30 μL of chloroform, and vortex for 5–10 s.
7. After centrifuging at 10,000*g* at 4°C, for 1 min, transfer 0.4 mL of the aqueous supernatant to a microfuge tube. Do not take any bacterial debris.
8. Add 0.4 mL of λ diluent containing 20% (w/v) polyethylene glycol 8000 and 2 *M* NaCl. Mix by vortexing, and incubate for 1 h on ice.
9. Centrifuge at 10,000*g* for 10 min at 4°C. Remove the supernatant, leaving 30–50 μL behind. Recentrifuge briefly to bring the liquid on the walls of the tube to the bottom, and remove the remaining supernatant.
10. Add 100 μL of extraction buffer, dissolve the phage pellet by vortexing, and incubate for 10 min at 68°C.
11. Extract the solution with 100 μL of phenol:chloroform, and recover the phage DNA by ethanol precipitation.
12. Dissolve the DNA in 100 μL of TE (pH 8.0).

3.3.2. Self-Circularization

1. Place 4 μL of each phage DNA solution from **Subheading 3.3.1.** in a microfuge tube. Add 6 μL of a *Not*I reaction mixture, and incubate at 37°C for 1 h and then at 70°C for 20 min.
2. Centrifuge the tubes briefly, and then transfer 2 μL of the *Not*I-digested DNA into a well of a U-bottomed, 96-well microtiter plate.
3. Add 5 μL of ligase reaction mixture. Mix by vortexing the plate, and incubate for 30 min at 16°C.
4. Add 20 μL of XL1-Blue competent cells directly to the ligated DNA, mix, and incubate on ice for 5 min. Heat for 90 s at 42°C, add 200 μL of 2X YT medium, and incubate for 30 min at 37°C with gentle shaking.
5. Transfer an appropriate volume (200 μL/90-mm plate or 50–100 μL/35-mm plate in a six-well plate) of the cell suspension onto an agar plate containing 100 mg/μL of ampicillin.
6. Incubate overnight (>10 h) at 37°C. Most of the clones thus obtained usually contain plasmids of the expected structure (i.e., pGEX-PUC-3T containing a cDNA between the *Sse*8387I sites), but it may be a good idea to pick up two colonies from each plate for backup.

3.4. Initial Characterization of Candidate Clones

First, cDNA clones from positive plaques need to be checked with respect to the following criteria: (1) Can the cDNA-encoded polypeptide really act as a substrate for the protein kinase? and (2) Does the polypeptide sequence represent at least a part of any naturally existing protein? The former point can be examined by in vitro phosphorylation assay of each of the purified GST-fusion proteins. The latter question arises since a polypeptide translated from a wrong reading frame or noncoding region of cDNA could be a good substrate by chance. Fortunately, clones that encode such artifactual products can be effectively excluded from the list of candidates by checking their product sizes on SDS-polyacrylamide gel electrophoresis (PAGE) (*see* **Subheadings 3.4.1.** and **Notes 1–8**).

3.4.1. Purification and Analysis of GST-Fusion Proteins Encoded by Candidate Clones

GST-fused recombinant proteins encoded by the rescued plasmids can be expressed in small bacterial culture, purified by GSH-agarose affinity beads, and then tested for phosphorylation by the protein kinase. An SDS-PAGE analysis of the phosphorylated GST fusions offers information about their sizes and extent of phosphorylation.

1. Pick a single colony and inoculate 3 mL of 2X YT medium containing 0.1% glucose and 100 μg/mL of ampicillin. Incubate overnight (12–18 h) at 37°C with vigorous shaking.

2. Place 0.15 mL of the overnight culture into a microfuge tube containing 50 μL of 60% sterile glycerol. Mix well and store at –70°C as a glycerol stock.
3. Place 1.35 mL of the overnight culture into a microfuge tube and prepare plasmid DNA by a mini-prep method suitable for DNA sequencing.
4. Add 7.5 μL of 100 m*M* IPTG to the remaining culture (1.5 mL), and incubate for 3 to 4 h at 37°C.
5. Transfer the culture into a microfuge tube, and centrifuge at 10,000*g* for 30 s at 4°C. Remove the supernatant and resuspend the bacterial pellet in 100 μL of ice-cold TE (pH 7.5).
6. Add 10 μL of freshly prepared lysozyme solution, mix, and leave on ice for 5 min.
7. Add 0.5 mL of TLB and lyse the cells by sonication. Centrifuge the tube at 12,000*g* for 15 min at 4°C and transfer the supernatant to a fresh tube.
8. Add 100 μL of 50% (v/v) GSH-agarose beads and incubate for 1 h at 4°C, with rocking.
9. Wash the beads twice with 0.5 mL of ice-cold TLB and once with 1 mL of MAPK reaction buffer (*see* **Subheading 3.2.3.**). Add to the washed beads 50 μL of the same buffer and mix by vortexing.
10. Transfer 20 μL of the suspension containing about 10 μL of the beads into a fresh tube, and add 5 μL of MAPK reaction buffer containing 250 μ*M* unlabeled ATP, 0.5 μCi of [γ-^{32}P]ATP, and 10–50 ng of ERK1 MAPK. Incubate the reaction mixture (25 μL) for 30–60 min at 30°C with occasional agitation.
11. Add 25 μL of 2X SB, boil for 3–5 min, and then analyze 10–30 μL of the samples by SDS-PAGE on a 12.5% polyacrylamide gel.
12. Stain the gel with Coomassie Brilliant Blue, dry, and expose to an X-ray film for autoradiography.

3.4.2. Sequence Analysis of Selected Clones

The 5'- and 3'-nucleotide sequences of the candidate clones can be determined using oligonucleotide primers designed to anneal just outside of the cloning sites. Amino acid sequences of the fusion proteins can be easily deduced from the 5'-nucleotide sequence. The nucleotide and amino acid sequences should be subjected to identity/homology searches using appropriate programs such as BLAST *(29)*. If a particular cDNA encodes a polypeptide sequence that is identical or similar to that of any known protein, this information would offer various hints for analysis of the protein as a substrate of the protein kinase. Some of them may be very plausible candidates for physiologic targets whereas some others may be most unlikely (e.g., secreted proteins). Nucleotide sequences of some clones may be found in an expressed sequence tag (EST) database, which may offer additional nucleotide sequence missing in the partial cDNA. Walking in EST databases via overlapping sequences often results in a long, contiguous sequence. The availability of several complete eukaryotic genome sequences and their increasingly well-annotated gene products should be of great value in defining the full-length coding region. If a consen-
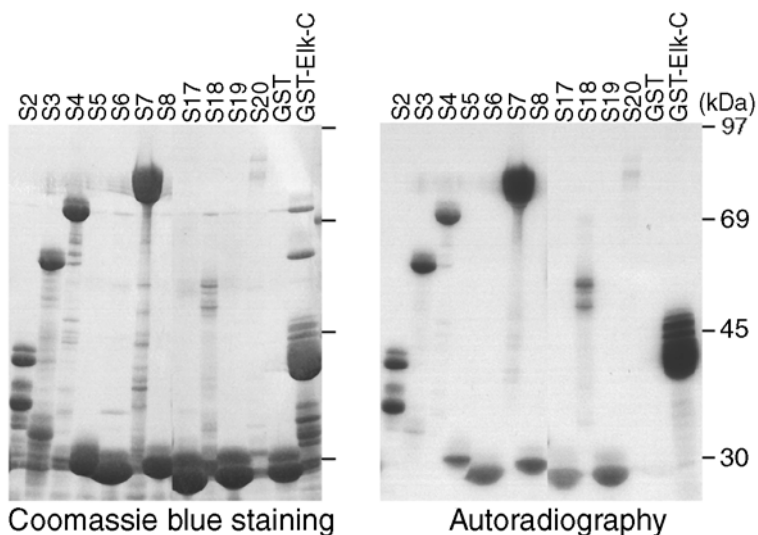
Fig. 5. In vitro phosphorylation analysis of positive clones in a screen for ERK1 substrates. Positive phage clones (S2–S8 and S17–S20) obtained in the screening were converted into plasmid clones, and then their GST-fusion proteins were expressed and purified using GSH-agarose. The resulting GST-fusion proteins together with GST and GST-Elk-C were phosphorylated by ERK1 MAPK in the presence of [γ-^{32}P]ATP and analyzed by SDS-PAGE. **(Left)** Coomassie blue staining of the gel. **(Right)** Autoradiograph of the same gel. Note that GST itself was not phosphorylated at all, whereas the artifactual recombinants (S5, S6, S8, S17, and S19) with similar small sizes and quantities were relatively weakly phosphorylated.

sus phosphorylation motif is known for the protein kinase in question, then it is worth scanning the predicted sequence of the cDNA product for potential phosphorylation sites *(3,30)*.

3.4.3. Characterization of Candidate ERK Substrates

In our screen for ERK1 substrates, we screened about 3×10^5 independent clones of a cDNA library prepared from HeLa cells, and obtained 120 positive clones from the secondary screening *(12)*. **Figure 5** shows a typical example of in vitro phosphorylation analyzed by SDS-PAGE. Although almost all of the recombinant proteins were phosphorylated by ERK1 MAPK in vitro, more than half of them were GST-proteins with a very short fusion partner (S5, S6, S8, S17, and S19). Sequencing analysis revealed that most of the extremely small fusion partners were artifactual products derived from out-of-frame ligation of cDNAs. Therefore, out of 120 clones, we selected 32 clones that expressed GST fusions larger than 32 kDa in total size (i.e., with a fusion partner of >5

kDa) for further characterization *(12)*. Every recombinant product of these 32 clones was a good in vitro substrate for ERK1 and is likely to represent some part of a naturally existing protein. Generalizing from this case, it is advisable to select only clones that produce a GST-fusion protein whose size is larger than, e.g., 32 kDa, which means that clones encoding cDNA-derived polypeptides of <5 kDa should be excluded from the substrate candidates. Although the frequency of appearance of such artifactual recombinants may depend on the protein kinase, it is probably desirable to exclude artifacts by checking the size of each GST-fusion protein prior to sequencing.

Sequencing analysis of the selected 32 candidates revealed that 14 clones corresponded to fragments of structurally known proteins, including two known physiologic ERK substrates, p90[RSK2] and c-Myc *(12)*. We also showed that one of the novel clones encoded a new MAPK-activated protein kinase and that this protein kinase, MNK1, is activated by ERK phosphorylation in vitro and also in response to ERK MAPK activation in vivo *(12)*. Although we have not characterized other clones in detail, most of them contained potential MAPK recognition sites, Ser-Pro or Thr-Pro, in their amino acid sequences. In another screen for cyclin E/Cdk2 substrates, 54 positive clones were identified from 1.0×10^6 independent clones of the HeLa cDNA library. At least two of them are likely to be in vivo Cdk substrates; one is caldesmon and the other is a novel protein, PRC1, which plays a role in cytokinesis *(14)*.

4. Notes

The phosphorylation screening approach is in principle generally applicable to all protein kinases, but the following issues should be considered.

1. A major problem that may arise in some cases is a high background caused by strong phosphorylation of an endogenous protein(s) derived from *E. coli* or λ phage. The severity of this background noise may depend on the substrate specificity of the protein kinase used. Although we did not encounter this problem in the screens for ERK1 MAPK and cyclin E/Cdk2 substrates, which are both proline-directed protein kinases, a preliminary experiment with the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase showed indistinguishable signals between positive and negative plaques. The high background problem may be overcome through development of a GSH-derivatized filter onto which the GST-fused recombinants can selectively be immobilized. The use of this affinity filter system should in principle reduce the background problem caused by bacterial proteins. No such GSH-filter is yet commercially available, but in preliminary experiments we have coupled GSH to cellulose filters and have found that the λGEX5/GSH-cellulose filter system worked well for ERK1. However, further development is required for practical, large-scale use.

2. Another strategy for reducing background problem relies on the fact that some protein kinases form a relatively stable complex with their substrates through a

domain distinct from the phosphorylation site. This is the case for MAPKs and their substrates *(31–41)*, and their upstream MAPK kinase activators *(38,42,43)*, which all possess a short basic motif that interacts with a docking site on the back side of the catalytic domain, and also the cyclin/Cdks, where substrates interact with a groove in the cyclin via a Z-Arg-X-Leu motif, where Z and X are usually basic *(44–47)*. This phenomenon might be of utility in selecting candidate clones for further study out of a large number generated by phosphorylation screening (see below), but it can in principle also be taken advantage of in a modified screening method. In this alternative protocol, plaque filters are first preincubated with the protein kinase in the absence of ATP to allow the kinase to bind recombinant substrates (this is analogous to the substrate-binding screen carried out with kinase-inactive cyclin E/Cdk2 reported by Zhao et al. *[4]*). The filters are then briefly washed to remove the excess, unbound protein kinase (different stringencies of washing could usefully be tried), and subsequently incubated in the presence [γ-^{32}P]ATP to allow phosphotransfer to occur within the kinase-substrate complex. We have not tested this alternative method, but it may reduce background signals derived from nonspecific phosphorylation and would be a way of identifying substrates that have a high-affinity binding site. In fact, phosphorylation screening may work well because, with the relatively low protein kinase concentration used in the screen, only high-affinity substrates that can bind via a docking site are phosphorylated efficiently.

3. Application of phosphorylation screening to protein-tyrosine kinases may be bedeviled by the high background problem because, in general, protein-tyrosine kinases are rather nonspecific in vitro. The use of antiphosphotyrosine antibodies to screen the phosphorylated λgt11 cDNA expression library selecting only the strongest signals has proved to be a successful way of identifying Src protein-tyrosine kinase substrates *(17,48)* An alternative solution would be to use an additional far-Western selection step utilizing a phosphotyrosine-binding domain. For example, plaque filters would first be incubated with a protein-tyrosine kinase in the presence of unlabeled ATP. After washing, the filters would next be incubated with an epitope-tagged or radiolabeled protein containing a particular Sre homology 2 (SH2) or phosphotyrosine-binding (PTB) domain to allow them to bind, and then positive plaques would be visualized by immunodetection or autoradiography. This protocol can act as a substrate screen based not only on the phosphorylating specificity of the protein kinase but also on the binding specificity of the phosphotyrosine-binding modules used. In fact, Kavanaugh et al. *(49)* showed that a screen of a λgt11 cDNA library with solid-phase phosphorylation by platelet-derived growth factor receptor tyrosine kinase followed by binding of ^{32}P-labeled Shc phosphotyrosine-binding domain resulted in cloning of a c-ErbB2 (Neu) cDNA, which contained tyrosine-phosphorylation-dependent phospho-tyrosine-binding sites.

4. Similar "double selection strategies" utilizing phosphorylation-dependent protein-protein interaction might be applicable also to some protein-serine/threonine kinases in combination with a protein module probe such as 14-3-3 proteins

(50), Pin1 *(51–53)*, FHA domain *(54)*, and the MPM-2 monoclonal antibody *(55)*, each of which specifically interacts with a phosphoamino acid-containing peptide motif. Indeed, a successful screen was carried out with MPM2 to identify substrates for mitotic protein kinases *(56,57)*.

5. Another undesirable source of background are GST fusion proteins that have strong (auto)phosphorylating activity or ATP-binding activity when they are isolated on the filter. To avoid this type of background, the filters are incubated in the presence of unlabeled ATP prior to the phosphorylation step. However, there may still be false positives of this sort. In this regard, other groups have found positive clones that contain Nm23 or Bc-NDPKIII nucleoside diphosphate kinases that utilize a phosphohistidine intermediate in phosphate transfer and have been reported to have protein-serine/threonine kinase activity in vitro *(58,59)*, when screening λGEX cDNA libraries.

6. As indicated above, if at all possible it is important to use a positive control phage in the screen. The use of either an intact substrate protein or a fragment that is known to be phosphorylated will make it easier to gage the intensity and shape of the autoradiographic signal that is to be expected from a positive clone in the library. This, combined with the use of two autoradiographic exposure times, should help the screener discriminate true-positive plaques, which will have rather dull, fuzzy signals within a certain size range, from false positives generated by pinpoint signals with a sharp rim observed only in the short exposure.

7. If a large number of clones is isolated in a screen, it will be necessary to decide which clone or subset of clones is to be characterized further. In the case of clones showing identity or homology to some known sequence, this information can provide the basis for this decision. In the case of novel proteins, however, it is not easy to choose one of the candidates. Therefore, some additional experiments need to be done for selection. As discussed earlier, protein kinases often interact with their substrates through docking sites that are independent of the phosphorylation sites, and, therefore, one selection criterion would be to test whether each GST-fused substrate protein can bind the protein kinase in an in vitro binding assay. If a substrate physically associates with the kinase to form a complex, it may be worth analyzing further. Alternatively, analysis of expression patterns of the candidates by Northern or *in situ* hybridization may be of utility. If a clone shows a characteristic expression pattern, such as cell type–specific, cell cycle–dependent, developmentally regulated or stimulation-inducible expression, this may suggest some physiologic relationship between the protein kinase and substrate.

8. It is important to remember that a protein identified by phosphorylation screening remains simply an "in vitro" substrate until proven to be a physiologic target of the protein kinase used, and this issue can be clarified only by in vivo analysis of whether the protein is phosphorylated under conditions in which the protein kinase in question is known to be activated. The final goal is, of course, to elucidate the function of the newly identified substrates and the physiologic significance of their phosphorylation. Although there is no general strategy for this purpose, isolation and sequence determination of a full-length cDNA clone as

well as antibody preparation are essential. Various experiments such as overexpression or ectopic expression of wild-type and phosphorylation site mutants, knock-in mutation of phosphorylation sites in the chromosomal copy of the gene in cell lines or the germline, decreasing expression by antisense or RNAi methods, and inhibition of the protein function by antibody microinjection may suggest the in vivo roles of the protein in question. Additionally, it may be informative to examine the subcellular localization and kinetics of synthesis and degradation of the substrate protein. Further, determination of the sites phosphorylated in vitro and in vivo and mutation of these sites would help to elucidate the biologic significance of phosphorylation in regulating the function of the protein kinase target.

We anticipate that the phosphorylation screening technique will be applicable to many other types of protein kinase, when the necessary refinements are made to optimize the screen for a protein kinase of interest. Furthermore, combination of the solid-phase phosphorylation and protein microarray technologies *(11,60)* should allow large-scale, systematic screening for protein kinase targets in the future.

Acknowledgments

References

1. Songyang, Z., Blechner, S., Hoagland, N., et al. (1994) Use of an oriented peptide library to determine the optimal substrates of protein kinases. *Curr. Biol.* **4,** 973–982.
2. Songyang, Z., Carraway, K. L., Eck, M. J., et al. (1995) Catalytic specificity of protein-tyrosine kinases is critical for selective signalling. *Nautre* **373,** 536–539.
3. Songyang, Z. (2001) Analysis of protein kinase specificity by peptide libraries and prediction of in vivo substrates. *Meth. Enzymol.* **332,** 171–183.
4. Zhao, J., Dynlacht, B., Imai, T., Hori, T., and Harlow, E. (1998) Expression of NPAT, a novel substrate of cyclin E-CDK2, promotes S-phase entry. *Genes Dev.* **12,** 456–461.
5. Zhao, J., Kennedy, B. K., Lawrence, B. D., et al. (2000) NPAT links cyclin E-Cdk2 to the regulation of replication-dependent histone gene transcription. *Genes Dev.* **14,** 2283–2297.
6. Yang, X., Hubbard, E. J., and Carlson, M. (1992) A protein kinase substrate identified by the two-hybrid system. *Science* **257,** 680–682.
7. Waskiewicz, A., Flynn, A., Proud, C. G., and Cooper, J. A. (1997) Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. *EMBO J.* **16,** 1909–1920.

8. Shah, K., Liu, Y., Deirmengian, C., and Shokat, K. M. (1997) Engineering unnatural nucleotide specificity for Rous sarcoma virus tyrosine kinase to uniquely label its direct substrates. *Proc. Natl. Acad. Sci. USA* **94,** 3565–3570.

9. Liu, Y., Shah, K., Yang, F., Witucki, L., and Shokat, K. M. (1998) Engineering Src family protein kinases with unnatural nucleotide specificity. *Chem. Biol.* **5,** 91–101.

10. Habelhah, H., Shah, K., Huang, L., Burlingame, A. L., Shokat, K. M., and Ronai, Z. (2001) Identification of new JNK substrate using ATP pocket mutant JNK and a corresponding ATP analogue. *J. Biol. Chem.* **276,** 18,090–18,095.

11. Zhu, H., Klemic, J. F., Chang, S., et al. (2000) Analysis of yeast protein kinases using protein chips. *Nature Genet.* **26,** 283–289.

12. Fukunaga, R. and Hunter, T. (1997) MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. *EMBO J.* **16,** 1921–1933.

13. Garcia, J., Ye, Y., Arranz, V., Letourneux, C., Pezeron, G., and Porteu, F. (2002) IEX-1: a new ERK substrate involved in both ERK survival and ERK activation. *EMBO J.* **21,** 5151–5163.

14. Jiang, W., Jimenez, G., Wells, N. J., et al. (1998) PRC1: a human mitotic spindle-associated CDK substrate protein required for cytokinesis. *Mol. Cell* **2,** 877–885.

15. Obata, T., Yaffe, M. B., Leparc, G. G., et al. (2000) Peptide and protein library screening defines optimal substrate motifs for AKT/PKB. *J. Biol. Chem.* **275,** 36,108–36,115.

16. Li, F., Adam, L., Vadlamudi, R. K., et al. (2002) p21-activated kinase 1 interacts with and phosphorylates histone H3 in breast cancer cells. *EMBO Rep.* **3,** 767–773.

17. Lock, P., Abram, C. L., Gibson, T., and Courtneidge, S. A. (1998) A new method for isolating tyrosine kinase substrates used to identify Fish, an SH3 and PX domain-containing protein, and Src substrate. *EMBO J.* **17,** 4346–4357.

18. Smith, D. B. (1993) Purification of glutathione S-transferase fusion proteins. *Meth. Mol. Cell. Biol.* **4,** 220–229.

19. Valtorta, F., Schiebler, W., Jahn, R., Ceccarelli, B., and Greengard, P. (1986) A solid-phase assay for the phosphorylation of proteins blotted on nitrocellulose membrane filters. *Anal. Biochem.* **158,** 130–137.

20. Glover, C. V. C. and Allis, C. D. (1991) Enzyme activity dot blots for assaying protein kinases. *Meth. Enzymol.* **200,** 85–90.

21. Young, R. A. and Davis, R. W. (1983) Efficient isolation of genes by using antibody probes. *Proc. Natl. Acad. Sci. USA* **80,** 1194–1198.

22. Huynh, T. V., Young, R. A., and Davis, R. W. (1985) Constructing and screening cDNA libraries in lambda gt10 and lambda gt11, in *DNA Cloning: A Practical Approach* (Glover, D. M., ed.), IRL, Oxford, UK, pp. 49–78.

23. Ausubel, F. M., Brent, R., Kingston, R. E., et al. (1997) *Current Protocols in Molecular Biology (Supplement 37),* John Wiley & Sons, NY.

24. Sambrook, I., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual,* 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

25. Bullock, W. O., Fernandez, J. M., and Short, J. M. (1987) XL1-Blue: a high efficiency plasmid transforming recA Escherichia coli strain with beta-galactosidase selection. *BioTechniques* **5,** 376–379.

26. Marais, R., Wynne, J., and Treisman, R. (1993) The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell* **73,** 381–393.

27. Williams, N. G., Paradis, H., Agarwal, S., Charest, D. L., Pelech, S. L., and Roberts, T. M. (1993) Raf-1 and p21[v-ras] cooperate in the activation of mitogen-activated protein kinase. *Proc. Natl. Acad. Sci. USA* **90,** 5772–5776.

28. Short, J. M., Fernandez, J. M., Sorge, J. A., and Huse, W. D. (1988) Lambda ZAP: a bacteriophage lambda expression vector with in vivo excision properties. *Nucleic Acids Res.* **16,** 7583–7600.

29. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215,** 403–410.

30. Yaffe, M. B., Leparc, G. G., Lai, J., Obata, T., Volinia, S., and Cantley, L. C. (2001) A motif-based profile scanning approach for genome-wide prediction of signaling pathways. *Nat. Biotechnol.* **19,** 348–353.

31. Kallunki, T., Su, B., Tsigelny, I., et al. (1994) JNK2 contains a specificity-determining region responsible for efficient c-Jun binding and phosphorylation. *Genes Dev.* **8,** 2996–3007.

32. Kallunki, T., Deng, T., Hibi, M., and Karin, M. (1996) c-Jun can recruit JNK to phosphorylate dimerization partners via specific docking interactions. *Cell* **87,** 929–939.

33. Gupta, S., Barrett, T., Whitmarsh, A. J., et al. (1996) Selective interaction of JNK protein isoforms with transcription factors. *EMBO J.* **15,** 2760–2770.

34. Galanis, A., Yang, S. H., and Sharrocks, A. D. (2001) Selective targeting of MAPKs to the ETS domain transcription factor SAP-1. *J. Biol. Chem.* **276,** 965–973.

35. Gavin, A. C. and Nebreda, A. R. (1999) A MAP kinase docking site is required for phosphorylation and activation of p90(rsk)/MAPKAP kinase-1. *Curr. Biol.* **9,** 281–284.

36. Gavin, A. C., Ni Ainle, A., Chierici, E., Jones, M., and Nebreda, A. R. (1999) A p90(rsk) mutant constitutively interacting with MAP kinase uncouples MAP kinase from p34(cdc2)/cyclin B activation in Xenopus oocytes. *Mol. Biol. Cell* **10,** 2971–2986.

37. Sharrocks, A. D., Yang, S. H., and Galanis, A. (2000) Docking domains and substrate-specificity determination for MAP kinases. *Trends Biochem. Sci.* **25,** 448–453.

38. Tanoue, T., Adachi, M., Moriguchi, T., and Nishida, E. (2000) A conserved docking motif in MAP kinases common to substrates, activators and regulators. *Nat. Cell Biol.* **2,** 110–116.

39. Tanoue, T., Maeda, R., and Adachi, M. (2001) Identification of a docking groove on ERK and p38 MAP kinases that regulates the specificity of docking interactions. *EMBO J.* **20,** 466–479.

40. Yang, S. H., Whitmarsh, A. J., Davis, R. J., and Sharrocks, A. D. (1998) Differential targeting of MAP kinases to the ETS-domain transcription factor Elk-1. *EMBO J.* **17,** 1740–1749.

41. Yang, S. H., Yates, P. R., Whitmarsh, A. J., Davis, R. J., and Sharrocks, A. D. (1998) The Elk-1 ETS-domain transcription factor contains a mitogen-activated protein kinase targeting motif. *Mol. Cell. Biol.* **18,** 710–720.

42. Bardwell, L., Cook, J. G., Chang, E. C., Cairns, B. R., and Thorner, J. (1996) Signaling in the yeast pheromone response pathway: specific and high-affinity interaction of the mitogen-activated protein (MAP) kinases Kss1 and Fus3 with the upstream MAP kinase kinase Ste7. *Mol. Cell. Biol.* **16,** 3637–3650.

43. Fukuda, M., Gotoh, Y., and Nishida, E. (1997) Interaction of MAP kinase with MAP kinase kinase: its possible role in the control of nucleocytoplasmic transport of MAP kinase. *EMBO J.* **16,** 1901–1908.

44. Schulman, B. A., Lindstrom, D. L., and Harlow, E. (1998) Substrate recruitment to cyclin-dependent kinase 2 by a multipurpose docking site on cyclin A. *Proc. Natl. Acad. Sci. USA* **95,** 10,453–10,458.

45. Adams, P. D., Sellers, W. R., Sharma, S. K., Wu, A. D., Nalin, C. M., and Kaelin, W. G. (1996) Identification of a cyclin-cdk2 recognition motif present in substrates and p21-like cyclin-dependent kinase inhibitors. *Mol. Cell. Biol.* 6623–6633.

46. Adams, P. D., Li, X., Sellers, W. R., et al. (1999) Retinoblastoma protein contains a C-terminal motif that targets it for phosphorylation by cyclin-cdk complexes. *Mol. Cell. Biol.* **19,** 1068–1080.

47. Takeda, D. Y., Wohlschlegel, J. A., and Dutta, A. (2001) A bipartite substrate recognition motif for cyclin-dependent kinases. *J. Biol. Chem.* **276,** 1993–1997.

48. Yokoyama, K., Su, I.-H., Tezuka, T., et al. (2002) BANK regulates BCR-induced calcium mobilization by promoting tyrosine phosphorylation of IP$_3$ receptor. *EMBO J.* **21,** 83–92.

49. Kavanaugh, W. M., Turck, C. W., and Williams, L. T. (1995) PTB domain binding to signaling proteins through a sequence motif containing phosphotyrosine. *Science* **268,** 1177–1179.

50. Muslin, A. J., Tanner, J. W., Allen, P. M., and Shaw, A. S. (1996) Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell* **84,** 889–897.

51. Ranganathan, R., Lu, K. P., Hunter, T., and Noel, J. P. (1997) Structural and functional analysis of the mitotic rotamase Pin1 suggests substrate recognition is phosphorylation dependent. *Cell* **89,** 875–886.

52. Shen, M., Stukenberg, P. T., Kirschner, M. W., and Lu, K. P. (1998) The essential mitotic peptidyl-prolyl isomerase Pin1 binds and regulates mitosis-specific phosphoproteins. *Genes Dev.* **12,** 706–720.

53. Lu, P. J., Zhou, X. Z., Shen, M., and Lu, K. P. (1999) Function of WW domains as phosphoserine- or phosphothreonine-binding modules. *Science* **283,** 1325–1328.

54. Durocher, D., Henckel, J., Fersht, A. R., and Jackson, S. P. (1999) The FHA domain is a modular phosphopeptide recognition motif. *Mol. Cell* **4,** 387–394.

55. Davis, F. M., Tsao, T. Y., Fowler, S. K., and Rao, P. N. (1983) Monoclonal antibodies to mitotic cells. *Proc. Natl. Acad. Sci. USA* **80,** 2926–2930.

56. Westendorf, J. M., Rao, P. N., and Gerace, L. (1994) Cloning of cDNAs for M-phase phosphoproteins recognized by the MPM2 monoclonal antibody and determination of the phosphorylated epitope. *Proc. Natl. Acad. Sci. USA* **91,** 714–718.

57. Matsumoto-Taniura, N., Pirollet, F., Monroe, R., Gerace, L., and Westendorf, J. M. (1996) Identification of novel M phase phosphoproteins by expression cloning. *Mol. Biol. Cell* **7,** 1455–1469.
58. Engel, M., Veron, M., Theisinger, B., et al. (1995) A novel serine/threonine-specific protein phosphotransferase activity of Nm23/nucleoside-diphosphate kinase. *Eur. J. Bioch≠em.* **234,** 200–207.
59. Matsushita, Y., Suzuki, T., Kubota, R., et al. (2002) Isolation of a cDNA for a nucleoside diphosphate kinase capable of phosphorylating the kinase domain of the self-incompatility factor SRK of Brassica campestris, *J Exp. Bot.* **53,** 765–767.
60. MacBeath, G. and Schreiber, S. L. (2000) Printing proteins as microarrays for high-throughput function determination. *Science* **289,** 1760–1763.

16

Study of Substrate Specificity of MAPKs Using Oriented Peptide Libraries

Michael B. Yaffe

1. Introduction

Determining in vivo substrates phosphorylated by different mitogen-activated protein kinases (MAPKs) is critical to understanding how these enzymes regulate cell division, differentiation, and growth. The evolution of new technologies, particularly mass spectroscopy, has greatly facilitated this analysis *(1–3)*, although usually the investigator must still perform large scale biochemical purification of the presumed substrate to determine its identity. The use of oriented peptide library screening combined with bioinformatics offers an alternative approach to predicting likely substrates of protein kinases through motif identification and database searching *(4–9)*. In this approach, degenerate peptide libraries containing an orienting Ser (or Thr) residue are phosphorylated in vitro by the MAPK of interest, and the subset of phosphorylated peptides is separated from the bulk of nonphosphorylated peptides by immobilized metal affinity chromatography (IMAC) *(10–12)*. The recovered phosphopeptides are then sequenced by Edman degradation in bulk, and selection for each amino acid in positions flanking the fixed Ser or Thr is determined. The end result of this process is a matrix of selection values that describes the optimal phosphorylation motif for the MAPK that was investigated. These matrices can then be used to search protein sequence databases and identify potential substrates that contain the best matches to the optimal MAPK phosphorylation motif. The putative substrates can then be examined in vitro and in vivo to determine whether they are in fact MAPK substrates under physiologic conditions. An basic outline of the technique is presented in **Fig. 1**.
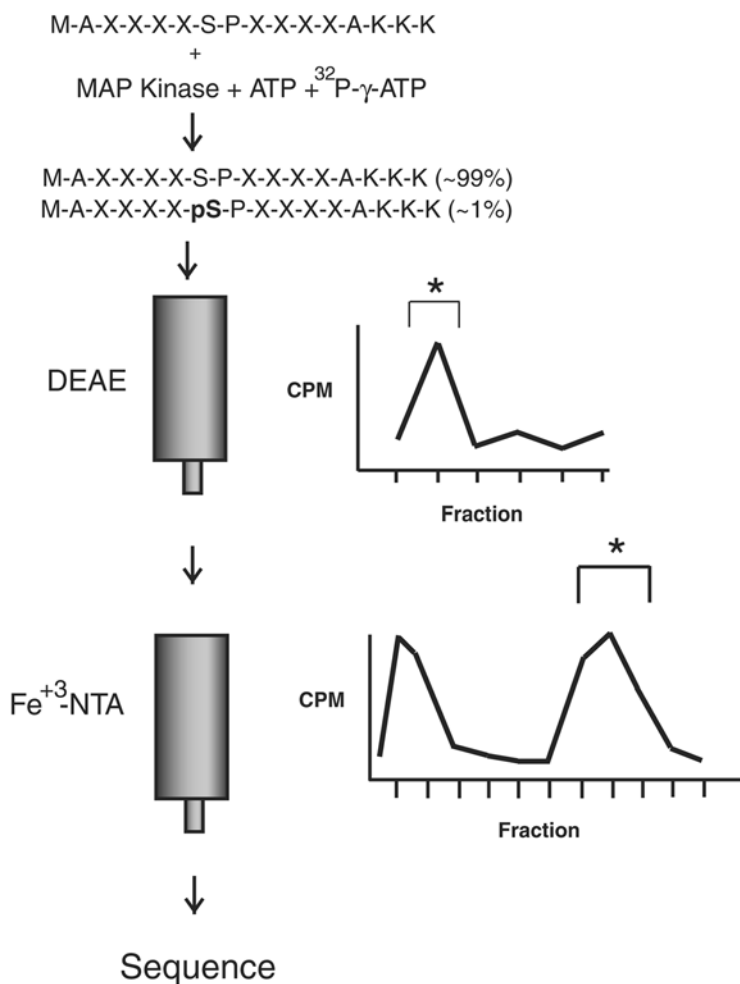
Fig. 1. Schematic of oriented peptide library screening procedure for MAPKs.

2. Materials

2.1. Chemicals

1. Standard reagents for peptide synthesis and protein sequencing.
2. DEAE Sephacel (#I6505; Sigma).
3. 30% Acetic acid in H_2O.
4. 0.1 M Acetic acid in H_2O.
5. 85% Phosphoric acid.
6. 10 mM Adenosine triphosphate (ATP), pH 7.0 (store at –20°C).
7. ^{32}P-γ-ATP (specific activity of 3000 Ci/mmol, 10 mCi/ml).

8. 100 m*M* FeCl$_3$ in 0.1 *M* acetic acid (prepare fresh).
9. 0.1 *M* EDTA, pH 8.0.
10. 0.1% Ammonium acetate, pH 8.0, freshly prepared and stored at 4°C.
11. 0.1% Ammonium acetate, pH 9.5, freshly prepared and stored at 4°C.
12. 0.1% Ammonium acetate, pH 11.5, freshly prepared and stored at 4°C.
13. Degenerate Ser- or Thr-containing peptide libraries (see below).
14. Nitrilotriacetic acid agarose (NTA Agarose), metal free (#N9153; Sigma).
15. Enzymatically active MAPK of interest to be screened (*see* **Note 1**).

2.2. Equipment

1. Peptide synthesizer (Applied Biosystems 431A) and protein sequencer (Applied Biosystems Procise, single- or multicartridge instrument, or Applied Biosystems 477A) and 12- and 3-mL polypropylene columns with tip plugs (Bio-Rad Poly-Prep Chromatography columns, #731-1550 and #732-6008; Bio-Rad).
2. P81 paper.
3. Scintillation counter.
4. Ring stand with clamps.
5. Speed-Vac.
6. Microcentrifuge.
7. Microcentrifuge tubes.
8. Disposable 50- and 15-mL polypropylene centrifuge tubes (#430291 and #430052 from Corning, or equivalent).

3. Methods

3.1. Design and Synthesis of Degenerate Peptide Libraries

Selection of the one or more residues around which to orient a degenerate peptide library is critical and determines the success or failure of the technique. In general, all peptide libraries used in the initial screening of a protein kinase should contain a single, fixed Ser residue, flanked by additional fixed and degenerate residues as necessary. It has been our experience that substitution of Thr for Ser in the orienting position has no effect on the optimal phosphorylation motif selected by the protein kinase, but Thr tends to perform somewhat less robustly as a general kinase substrate. Consequently, we do not recommend that Thr-oriented libraries be used in the initial determination of kinase motifs, though they can be used during motif refinement experiments using secondary libraries (see below). Frequently, libraries containing only a single, fixed Ser residue are insufficiently phosphorylated by the kinase of interest to provide enough phosphopeptides for accurate analysis. In these cases, the inclusion of additional fixed amino acids besides Ser that are known to be important for phosphorylation usually suffices to provide an adequate kinase substrate for motif determination (*see* **Note 2**). For proline-directed MAPKs, e.g., this is accomplished by constructing libraries containing a fixed

Ser-Pro sequence. In cases in which the information about additional amino acid selection beyond Ser is unknown, it is useful to perform the kinase assay using several different libraries containing an assortment of fixed residues in addition to the orienting Ser position (i.e., libraries containing a fixed Arg residue position amino-terminal to the Ser, or an Ile residue one position carboxyl-terminal to the Ser residue).

Libraries containing eight or fewer degenerate positions work best for initial screening. These libraries already have more than 25 billion degenerate peptide combinations, and one can position the fixed amino acid residue within the center of the degenerate stretch, or along either end. Incorporating >10 degenerate positions in the starting library will increase the initial complexity of the peptide mixture; however, it can simultaneously decrease the relative fraction of peptides within the starting mixture that are phosphorylated, yielding inadequate amounts for sequencing. Longer stretches of degenerate positions are possible, however, in secondary libraries (see below). Libraries are routinely constructed beginning with the sequence Met-Ala and terminated after the last degenerate position with the sequence Ala-Lys-Lys-Lys. A typical library used to deduce the phosphorylation motif for MAPKs, e.g., contains the sequence Met-Ala-Xxx-Xxx-Xxx-Xxx-Ser-Pro-Xxx-Xxx-Xxx-Xxx-Ala-Lys-Lys-Lys, in which Xxx indicates all amino acids except Cys, Ser, Thr, and Tyr. The Met-Ala sequence at the N-terminus of the peptide libraries allows verification that peptides from this mixture are being sequenced, and provides a quantitative estimate of peptide binding. The N- and C-terminal Ala residues bracketing the degenerate positions allow estimation of how much peptide has been lost during sequencing (i.e., repetitive yield) and gives an indication of cleavage lag and carryover between sequencing cycles. The polylysine tail ensures solubility of the libraries and aids in retention of the peptides on the Biobrene-coated filters during the sequencing steps. The omission of Ser, Thr, and Tyr from the degenerate positions forces phosphorylation of the library by the MAPK of interest to occur only at the position of the orienting Ser residue within the degenerate sequence motif.

Once an initial binding motif has been determined, it is often helpful to construct additional secondary libraries to further refine and expand individual amino acid preferences. For example, an initial screening of MAPKs using a Ser-Pro-based library might reveal a strong preference for Pro in the Ser–2 position (PXSP motif). One should then construct a secondary library in which Pro residues are fixed in both the Ser–2 and Ser+1 positions, containing the general sequence Met-Ala-Xxx Xxx- Xxx- Xxx-Pro- Xxx-Ser-Pro- Xxx- Xxx-Xxx- Xxx-Ala-Lys-Lys-Lys. Since the general affinity of the kinase for any random peptide within this Pro-Xxx-Ser-Pro library is higher than that for a peptide from the initial Ser-Pro library, weaker interactions from residues

within and bracketing the Pro-Xxx-Ser-Pro core will now dominate the selectivity. In the initial screening performed with the Ser-Pro-only library, information about these interactions was partially lost, since the binding was largely dominated by selection for Pro in the Ser–2 position.

Solid-phase synthesis of the degenerate peptide libraries is performed using a Rink amide resin matrix and an automated peptide synthesizer (Applied Biosystems 431A) according to standard BOP/HOBt coupling protocols with commercially available Fmoc-protected amino acids. At the degenerate positions, equal amounts of Fmoc-blocked amino acids (except Cys, Ser, Thr, and Tyr) are weighed out and loaded into the synthesizer cartridges so that the mixture is in fourfold molar excess to the coupling resin. The ratio of Fmoc-blocked amino acids needs to be adjusted slightly on different synthesizers in order to obtain a roughly even distribution of degenerate amino acids. Synthesis of oriented degenerate peptide libraries can also be performed by an outside peptide synthesis facility for a reasonable cost. One such supplier is the Tufts University core facility www.tucf.com.

Once the synthesis is complete, the degenerate peptide libraries are deprotected and cleaved from the resin for 3 h at room temperature using 4 mL of a cleavage reagent prepared by dissolving 0.75 g of phenol in 10 mL of trifluoroacetic acid, and adding 0.5 mL of H_2O, 0.5 mL of thioanisole, and 0.25 mL of ethane dithiol. The crude library mixture is precipitated by slow addition into 50 mL of diethyl ether followed by cooling to –20°C for 1 h. The precipitate is filtered using a fretted funnel, washed six times with chilled diethyl ether, dissolved in H_2O, and lyophilized. The final peptide libraries are stored as dry powders at –20°C.

Working stocks of the libraries are prepared at 10–30 mg/mL in either phosphate-buffered saline or the kinase buffer of interest and adjusted by the addition of dry HEPES or Tris to pH ~7.0. A small amount of the library is sequenced to ensure that all amino acids are present at similar amounts (within a factor of three) in each degenerate position.

3.2. Phosphorylating the Oriented Degenerate Peptide Library by MAPKs

The MAPK of interest should be incubated with 1 mg of the degenerate peptide library mixture until 0.2–1% of the library has been phosphorylated. For a typical peptide library with an average Mw of ~2000 Daltons, this corresponds to ~1–5 nmol of phosphorylated product, which is further purified as described in **Subheadings 3.3.** and **3.4.**

1. In a 1.5-μL microfuge tube add the following:
   a. 30 μL of 10X kinase buffer. (Use whatever buffer the kinase prefers for phosphorylation of a known substrate. For many MAPKs, a good 10X buffer is 200 m*M* HEPES, pH 7.5, and 100 m*M* MgCl$_2$.)

b. 1 mg of the degenerate peptide library mixture (100 μL of a 10 mg/mL solution or 33 μL of a 30 μg/mL solution).

c. 3 μL of 10 m*M* ATP.

d. x μL of purified active MAPK (typically 5–25 μL).

e. y μL of double distilled deionized H_2O to bring the final volume to 300 μL.

2. Incubate the kinase reaction at 30°C. Determine the extent of library phosphorylation by removing a 5-μL sample of the reaction mixture at 0, 1, and 2 h and spotting it onto labeled 2 × 2 cm squares of P81 paper. Allow the squares to air-dry, and then wash the squares four times in a beaker containing 50 mL of 0.45% phosphoric acid for 2 min/wash. Place the squares in scintillation vials, cover with 5–10 mL of scintillation fluid, and count.

3. Based on the P81-bound counts and the specific activity of the ^{32}P-γ-ATP on the day the assay was performed, it is a straightforward matter to determine the amount of phosphorylated peptide. Each 300-μL reaction contains a total of 30 nmol of ATP into which the trace amount of radioactive ATP is distributed. Subtract the counts per minute incorporated in the 0-h background control from the 1- and 2-h samples, and multiply by 60 (= 300 μL total/5 μL counted) to obtain the total counts per minute incorporated into the peptide library. To determine the total nanomoles of phosphorylated peptide library, use the following equation:

nmols phosphopeptide=[(cpm _incorporated)/(total ^{32}P cpm in reaction)] × 30

For example, assuming a specific activity of 10 mCi/mL and a counting efficiency of 100%, 1 nmol of ATP incorporation into the peptide library mixture would give a total of 7400 cpm incorporated. It may be necessary to allow the kinase reaction to proceed overnight to obtain a sufficient amount of phosphorylated product.

4. Stop the reaction by adding 30% acetic acid to a final concentration of 15%.

3.3. Removal of Unincorporated ATP and Separation of Phosphorylated From Nonphosphorylated Peptides

The peptide library mixture is partially purified from the unincorporated ATP using DEAE-Sephacel, and the phosphorylated peptides separated from their nonphosphorylated counterparts using an immobilized Fe^{3+} column.

1. Prepare the DEAE-Sephacel resin by placing 3 mL of the slurry and 10 mL of 30% acetic acid in a 50-mL disposable polypropylene centrifuge tube (#430291 from Corning or equivalent). Mix gently, and then pellet the resin by low-speed centrifugation (250*g* for 2 min). Discard the supernatant and resuspend the washed resin in 1.5 mL of 30% acetic acid.

2. Clamp a disposable 12-mL chromatography column in a ring stand, keeping the tip plugged. Fill the column with 8 mL of 30% acetic acid and add 1.5 mL of the washed, resuspended DEAE-Sephacel slurry. Allow the resin to settle for 20 min by gravity, giving a packed bed volume of ~1 mL.

3. Number seven microfuge tubes 1–7. Using a marker, indicate the position of 600 μL on tube 1, 1 mL on tube 2, and 500 μL on tubes 3–7 on the sides of the tubes.

4. Remove the plug from the column, and allow the column to run until the top of the resin is just exposed. Apply the phosphorylated library mixture to the top of the resin. Allow the column to run by gravity, collecting the first 600 μL of eluent (the dead volume) into tube 1. Switch to tube 2 and add an additional 600 μL of 30% acetic acid to the top of the DEAE column. Collect the effluent in tube 2. Finally, add 3 mL of 30% acetic acid to the top of the column, and collect the next 400 μL into tube 2 so that the final volume in this tube is 1 mL. Collect 500-μL fractions into tubes 3–7.

5. Count 5 μL of each fraction on a scintillation counter, and then calculate the total counts per minute contained in each fraction. Plot the counts per minute as a function of fraction number. The phosphorylated peptide library should elute in fraction 2, while the unincorporated ATP generally elutes in fractions 5–7 and later.

6. Dry down the fraction containing the peptide library (fraction 2) overnight on a Speed-Vac apparatus. At this point the sample can be stored at –20°C prior to proceeding to the next step.

7. Clamp a disposable 3-mL chromatography column in a ring stand, keeping the tip plugged. Fill the column with 2 to 3 mL of H_2O, add 900 μL of a 1:1 slurry of the NTA Agarose, and allow the resin to settle by gravity (*see* **Note 3**).

8. Remove the tip plug from the column, and allow the covering H_2O to drain until the surface of the resin is exposed. Strip the resin by washing the column sequentially with 4 mL of H_2O, 2 mL of 0.1 M EDTA, 4 mL of H_2O, and 2 mL of 0.1 M acetic acid.

9. Charge the resin by adding 2 mL of 100 mM $FeCl_3$ in 0.1 M acetic acid, followed by a single wash using 2 mL of 0.1 M acetic acid. The column should be yellow.

10. Prerun the column to elute any contaminants prior to sample loading. Wash the column sequentially with 2 mL of 0.1 M acetic acid; 1 mL of H_2O, 1 mL of 0.1% ammonium acetate, pH 8.0; 2 mL of 0.1% ammonium acetate, pH 9.5; 10 mL of H_2O; and 6 mL of 0.1 M acetic acid. The color of the column will change progressively from yellow to brown, and then back to light yellow again.

11. During the prerun step, resuspend the dried peptide library sample in 50 μL of 0.1 M acetic acid. Adjust the pH to ~3.5 using 30% acetic acid, and resuspend by aggressive vortexing.

12. Apply the sample to the Fe^{3+} column. Rinse the sample tube with another 50 μL of 0.1 M acetic acid, pH 3.5. A handheld scintillation counter is useful to ensure that most of the radioactivity, corresponding to the phosphorylated peptide library, has been adequately transferred to the column.

13. Number microcentrifuge tubes 1–10. Develop the column, collecting 0.5-mL fractions, by eluting with 1 mL of 0.1 M acetic acid (fractions 1 and 2); 1 mL of H_2O (fractions 3 and 4); 1 mL of 0.1% ammonium acetate, pH 8.0 (fractions 5 and 6); and 2 mL of 0.1% ammonium acetate, pH 11.5 (fractions 7–10). Count 5 μL of each fraction in a scintillation counter and plot the counts per minute as a function of fraction number. Most of the radioactivity should elute in fractions 1 and 2 and 7–10. Fractions 1 and 2 correspond to nonpeptide-containing ^{32}P, whereas fractions 7–10 correspond to the MAPK-phosphorylated peptides.

14. Pool the tubes containing the phosphorylated peptide library (generally fractions 7–9) into a single tube and dry down in a Speed-Vac apparatus. Dissolve the

pellet in 250–500 μL of H_2O and relyophilize several times to remove as much volatile salt as possible. It may be necessary to add small amounts of 30% acetic acid to neutralize the ammonium acetate and resuspend the sample, as evidenced by release of radioactivity into the liquid phase. Resuspend the final pellet in 80 μL of H_2O and sequence 40 μL by automated Edman degradation.

3.4. Peptide Sequencing and Data Analysis

Samples can be sequenced by any commercial sequencing facility, using any sequencing protocol sufficient to detect individual amino acids at the 5- to 10-pmol range. It is necessary to sequence the initial peptide library mixture as well. In our laboratory, the peptide libraries are spotted onto Biobrene-coated glass-fiber filter discs and loaded into the cartridges of an Applied Biosystems Procise sequencer. A pulsed-liquid solvent delivery cycle is used with standard times for phenylisothiocyanate (PITC) coupling, extraction, cleavage, and transfer recommended by the manufacturer, along with a 9-min phenylthio-dantoin (PTH) conversion at 64°C. The PTH-derivitized amino acids are then detected by high-performance liquid chromatography (HPLC) with a gradient optimized for separation between amino acids, particularly Phe, Ile, Lys, and Leu, which elute late. The quantity of each amino acid in picomoles, within each sequencing cycle, is reported. For best accuracy, amino acid standards should be prepared weekly, and the calibration run preceding each sequencing reaction should be performed using amounts of each PTH amino acid reasonably close to what is expected for the actual peptide library being sequenced.

To determine the optimal peptide motif phosphorylated by the MAPK, the relative abundance of each amino acid at a given sequencing cycle of the phosphorylated peptide mixture is divided by the relative abundance of the same amino acid in that cycle from the starting library mixture. This ratio corrects for variations in the mol percentages of particular amino acids in the starting library as well as variations in yield of amino acid recoveries during sequencing. The calculations can easily be performed in spreadsheet fashion.

1. Let A_{ij} represent the amount, in pmoles, of amino acid i reported for the MAPK-phosphorylated library sample in sequencing cycle j. For example, A_{34} might represent the amount of Glu (i.e., letting $i = 3$ denote Glu) in sequencing cycle 4 ($j = 4$). Begin by calculating the mol percentage of each amino acid in one sequencing cycle (fixed j) that contains the degenerate mixture of amino acids. This value, denoted MP_{ij} (for sample <u>M</u>ol <u>P</u>ercentile), is given by

$$MP_{ij} = (A_{ij})/(\Sigma_{i=1}^{16} A_{ij})$$

in which the sum of A_{ij} in the denominator is performed over all 16 amino acids in that sequencing cycle j. Perform this same calculation for all remaining sequencing cycles $j + 1$, $j + 2$, and so on that contain degenerate amino positions.

2. Perform the identical calculations for the mol percentages of each amino acid present in the degenerate positions in the initial unphosphorylated peptide library mixture. Let B_{ij} represent the amount, in picomoles, of amino acid i reported for the starting library mixture in sequencing cycle j. The mol percentage of amino acid i in cycle j for the control, denoted by CP_{ij} (<u>C</u>ontrol <u>P</u>ercentile), is similarly given by

$$CP_{ij} = (B_{ij})/(\Sigma_{i=1}^{i=16} B_{ij})$$

3. Calculate the raw selectivity value for a given amino acid i in a particular sequencing cycle j, denoted S_{ij}^{raw}, by $S_{ij}^{raw} = MP_{ij}/CP_{ij}$.
4. Normalize the raw selectivity values so that the sum of all selectivity values in a given sequencing cycle j is equal to the total number of possible amino acids. If the library has been synthesized without Cys, Ser, Thr, or Tyr in the degenerate positions, then the sum of all selectivity values should be normalized to 16, by defining

$$S_{ij}^{norm} = [(S_{ij}^{raw})/(\sum_i S_{ij}^{raw})] \times 16$$

in which the calculation is performed for individual fixed values of j.

Graphic plots of normalized preference values vs amino acid for each sequencing cycle (**Fig. 2**) are very useful for revealing the optimal peptide motif. If the kinase has no specificity at a particular residue position within the motif, then the mol-percentage of all amino acids in this position will be the same as that present in the initial mixture, and the preference values for all amino acids will be ~1. In identifying amino acids that are selected at particular positions within a motif, one should pay special attention to those amino acids that change from cycle to cycle, rather than those that remain persistently slightly elevated or depressed in relative abundance. This latter effect usually results from small systematic errors in sequencing for a particular residue between the sample and the starting library mixture (*see* **Note 4**).

It is strongly recommended that individual peptides containing the optimal motif sequence, or containing the motif with amino acid substitutions at key selected positions, be synthesized and evaluated for phosphorylation by the kinase used in peptide library screening *(6,8)*. Measurements of k_m and V_{max} values for these peptides by the MAPK is important to validate the peptide library motif selection.

3.5. Using Optimal Domain Motif to Elucidate Protein Interactions and Cell Signaling Pathways

Once the phosphorylation motif for a particular MAPK has been determined, it can then be used to prospectively identify likely phosphorylation sites on known substrates. Alternatively, one can use the matrix of selectivity values to identify new potential MAPK substrates via searching in protein or translated

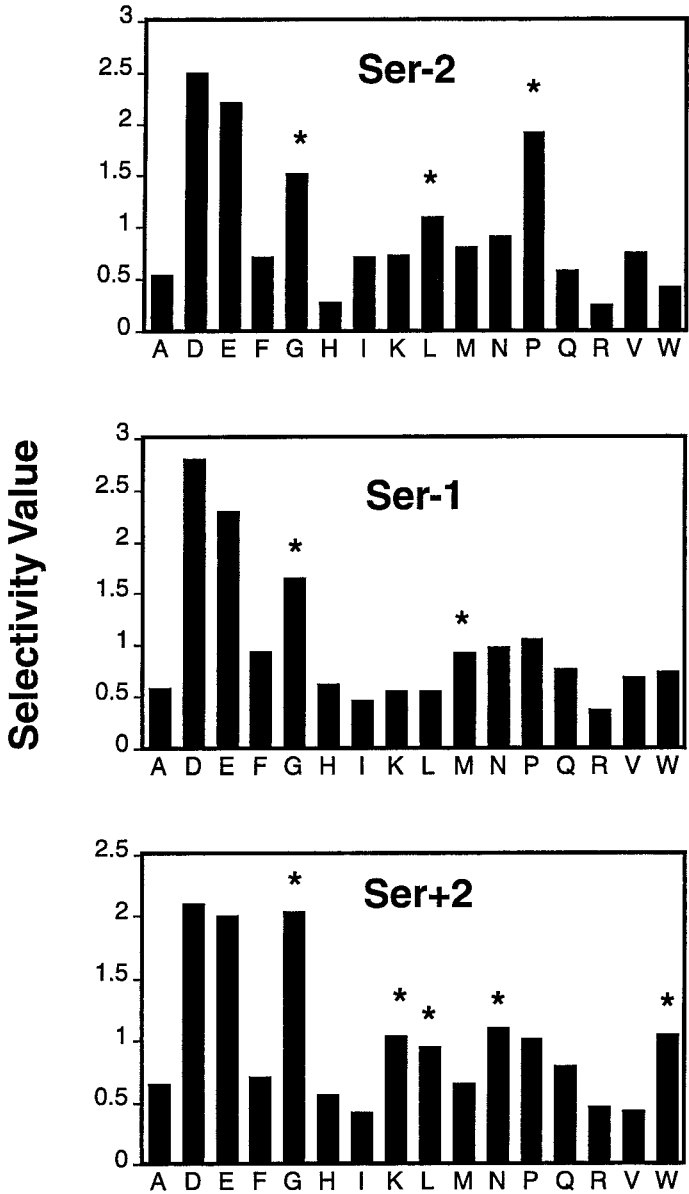Fig. 2. Graphic representation of amino acid selectivity data. The selectivity value for each amino acid at one particular sequencing cycle is shown. The data are from Erk2 screened with the oriented peptide library shown in **Fig. 1**. Asterisks denote selection values showing significant change from cycle to cycle. The optimal sequence motif for Erk2 from these data would be (P/L/G)-(M/G)-S-P-(G/N/K/L/W).

nucleic acid databases. In database searching it is important to allow for partial matches to the motif in scoring query sequences to avoid missing potential substrates, since many proteins will match the motif at some but not all residue positions. A variety of software available on the Internet can accept the matrix of selection values obtained from peptide library screening and search for proteins containing high scoring matches to the optimal motif. Relevant programs include PatScan (http://www-unix.mcs.anl.gov/compbio/PatScan/HTML/patscan.html) and Scansite *(9)*; the latter is specifically designed to accept user-input matrices for searching individual protein or protein database queries and reports a rank-ordered list of matching sites and putative substrates. One can also perform a restricted search for the motif in a subset of proteins having homology to a known target using Pattern Hit Initiated Blast (PHI-BLAST) *(13)*. Many bona fide MAPK substrates possess both potential phosphorylation sequences and sequences that correspond to MAPK docking sites *(14–17)*. Database scans that search for matches to both the phosphorylation motif and the docking site motif are likely to be significantly more accurate in predicting true MAPK substrates.

4. Notes

1. The MAPK of interest can be obtained by overexpression of the recombinant protein in either bacteria or eukaryotic cells. It is usually best to express the isolated kinase domain alone, often fused to an affinity tag such as gluthathione S-transferase or $(His)_6$ to facilitate purification. In some cases, the introduction of point mutations that convert phosphorylated Ser or Thr residues within the kinase activation loop to Asp are necessary to increase the intrinsic phosphotransferase activity of the enzyme. Alternatively, the recombinant protein can be phosphorylated in vitro by upstream activating MAPK kinases, or combinations of MAPK kinase kinases and MAPK kinases, followed by purification of the MAPK away from the upstream activator kinases. For many MAPKs, expression in insect cells (Sf9 cells) using baculoviruses, followed by standard isolation techniques (e.g., fast performance liquid chromatography [FPLC], affinity chromatography) is preferred. It is critically important to purify the MAPK kinase away from coassociating protein kinases to ensure that the activity being measured corresponds to the kinase of interest. Performing an identical peptide library screen using a catalytically inactive mutant form of the MAPK can serve as a useful control. The amount of MAPK required for the assay depends entirely on its specific activity. It has been our experience that microgram quantities are usually sufficient.
2. For a kinase whose motif is unknown, we typically perform initial trials using libraries that contain the following series of fixed and degenerate amino acids:
    a. 4S4 Library: Met-Ala-Xxx-Xxx-Xxx-Xxx-Ser-Xxx-Xxx-Xxx-Xxx-Ala-Lys-Lys-Lys

...

    b. 4SP4 Library: Met-Ala-Xxx-Xxx-Xxx-Xxx-Ser-Pro-Xxx-Xxx-Xxx-Xxx-Ala-Lys-Lys-Lys

    c. 4R2S4 Library: Met-Ala-Xxx-Xxx-Xxx-Xxx-Arg-Xxx-Xxx-Ser-Xxx-Xxx-Xxx-Xxx-Ala-Lys-Lys-Lys

    d. 4SQ4 Library: Met-Ala-Xxx-Xxx-Xxx-Xxx-Ser-Gln-Xxx-Xxx-Xxx-Xxx-Ala-Lys-Lys-Lys

    e. 4SF4 Library: Met-Ala-Xxx-Xxx-Xxx-Xxx-Ser-Phe-Xxx-Xxx-Xxx-Xxx-Ala-Lys-Lys-Lys

3. Alternatively, one can use Immobilized Iminodiacetic Acid Agarose (IDA Agarose) with 1,4 butanediol diglycidyl ether spacer (#20277; Pierce) instead of NTA Agarose. In this case, the sample should be resuspended in 50 mM MES (pH 5.5), 1 M NaCl (buffer A), and the Fe^{3+}-IDA column equilibrated with 2 mL of buffer A prior to sample loading.

4. Although the Fe^{3+} column separates phosphorylated from nonphosphorylated peptides, there is a background from nonphosphorylated peptides that contain large amounts of Asp and Glu in the degenerate positions and, consequently, adhere to the Fe^{3+} column. Therefore, great care should be used in assigning selectivity values to these two amino acids. The amount of nonphosphorylated peptide background can be estimated based on the amount of Ser in the fixed position of the phosphorylated library sample. (Phosphoserine, generated by kinase phosphorylation, undergoes β-elimination to form dithiothreitol adducts during sequencing, and consequently chromatographs at different positions from the unphosphorylated Ser residue.) The background estimate of nonphosphorylated peptides can be subtracted from the final sequenced peptide data prior to calculation of selectivity values, though this is not routinely performed unless the background signal exceeds ~30% of the sample signal.

Acknowledgments

References

1. Aitken, A. and Learmonth, M. (1997) Analysis of sites of protein phosphorylation. *Methods Mol. Biol.* **64,** 293–306.
2. Resing, K. A. and Ahn, N. G. (1997) Protein phosphorylation analysis by electrospray ionization-mass spectrometry. *Methods Enzymol.* **283,** 29–44.
3. Zhang, W., Czernik, A. J., Yungwirth, T., Aebersold, R., and Chait, B. T. (1994) Matrix-assisted laser desorption mass spectrometric peptide mapping of proteins separated by two-dimensional gel electrophoresis: determination of phosphorylation in synapsin I. *Protein Sci.* **3,** 677–686.
4. Wu, J., Ma, Q. N., and Lam, K. S. (1994) Identifying substrate motifs of protein kinases by a random library approach. *Biochemistry* **33,** 14,825–14,833.

5. Songyang, Z., Blechner, S., Hoagland, N., et al. (1994) Use of an oriented peptide library to determine the optimal substrates of protein kinases. *Curr. Biol.* **4,** 973–982.

6. Nishikawa, K., Toker, A., Johannes, F. J., Songyang, Z., and Cantley, L. C. (1997) Determination of the specific substrate sequence motifs of protein kinase C isozymes. *J. Biol. Chem.* **272,** 952–960.

7. Songyang, Z. and Cantley, L. C. (1998) The use of peptide library for the determination of kinase peptide substrates. *Methods Mol. Biol.* **87,** 87–98.

8. Obata, T., Yaffe, M. B., Leparc, G. G., et al. (2000) Peptide and protein library screening defines optimal substrate motifs for AKT/PKB. *J. Biol. Chem.* **275,** 36,108–36,115.

9. Yaffe, M. B., Leparc, G. G., Lai, J., Obata, T., Volinia, S., and Cantley, L. C. (2001) A motif-based profile scanning approach for genome-wide prediction of signaling pathways. *Nat. Biotechnol.* **19,** 348–353.

10. Muszynska, G., Andersson, L., and Porath, J. (1986) Selective adsorption of phosphoproteins on gel-immobilized ferric chelate. *Biochemistry* **25,** 6850–6853.

11. Holmes, L. D. and Schiller, M. R. (1997) Immobilized Iron(III) metal affinity chromatography for the separation of phosphorylated macromolecules: ligands and applications. *J. Liq. Chrom. Rel. Technol.* **20,** 123–142.

12. Neville, D. C., Rozanas, C. R., Price, E. M., Gruis, D. B., Verkman, A. S., and Townsend, R. R. (1997) Evidence for phosphorylation of serine 753 in CFTR using a novel metal-ion affinity resin and matrix-assisted laser desorption mass spectrometry. *Protein Sci.* **6,** 2436–2645.

13. Zhang, Z., Shaffer, A. A., Miller, W., et al. (1998) Protein sequence similarity searches using patterns as seeds. *Nucleic Acids Res.* **26,** 3986–3990.

14. Kallunki, T., Su, B., Tsigelny, I., et al. (1994) JNK2 contains a specificity-determining region responsible for efficient c-Jun binding and phosphorylation. *Genes Dev.* **8,** 2996–3007.

15. Dai, T., Rubie, E., Franklin, C. C., et al. (1995) Stress-activated protein kinases bind directly to the delta domain of c-Jun in resting cells: implications for repression of c-Jun function. *Oncogene* **10,** 849–855.

16. Jacobs, D., Glossip, D., Xing, H., Muslin, A. J., and Kornfeld, K. (1999) Multiple docking sites on substrate proteins form a modular system that mediates recognition by ERK MAP kinase. *Genes Dev.* **13,** 163–175.

17. Tanoue, T., Adachi, M., Moriguchi, T., and Nishida, E. (2000) A conserved docking motif in MAP kinases common to substrates, activators and regulators. *Nat. Cell Biol.* **2,** 110–116.

17

Ras Signaling Pathway for Analysis of Protein–Protein Interactions in Yeast and Mammalian Cells

Ami Aronheim

1. Introduction

The mitogen-activated protein kinase (MAPK) signaling pathway is initiated by diverse stimuli that occur at the cell membrane and result in a change in pattern of gene expression in the nucleus. Although multiple proteins are involved in the transmission process through a complex cascade of events, the signal is rapidly and efficiently transmitted *(1)*. Protein–protein interaction plays a major role in the transmission process itself as well as in the maintenance of the signaling molecules in an inactive dormant state within the cell. A large variety of mechanisms are involved in the translation of the signal into molecular events. One of the mechanisms involved is the generation of increased local concentration of reactant within a large compartment such as the cytoplasm. A case in point is the plasma membrane. Upon growth factor stimulation, e.g., the inner leaflet of the plasma membrane serves as a subcellular compartment for the recruitment of enzymes in close proximity to their substrates, thereby overcoming the thermodynamic barrier and permitting the enzymatic reaction to occur. The plasma membrane compartment can be subdivided into multiple microenvironments as well *(2)*. Protein recruitment *per se* is possibly accompanied by phosphorylation and a transient conformational change that results in potentiation of the corresponding enzymatic activity. Autophosphorylation of the cytoplasmic tail of growth factor receptors is the best example for this regulatory process, which serves as a docking platform for multiple effector molecules such as Grb2-Sos, phospholipase Cγ, p85-p110 subunits of phosphatidylinositol 3-kinase, GAP, and phosphotyrasine phosphatase (PTP). For many of these molecules, it has already been demonstrated that their direct recruitment to the plasma membrane results in activation of their enzymatic activity or their downstream signaling *(3–6)*. This is mainly

owing to the fact that the corresponding substrates for these signaling molecules are localized in the proximity of the plasma membrane. Interestingly, the members of the Ras superfamily of guanosin triphosphate (GTP)-binding proteins act as molecular switches in mediating multiple signal transduction pathways in response to a large variety of stimuli. The small GTPases are themselves localized to the plasma membrane via a conserved domain known as the CAAX box, located at the carboxy terminal *(7)*. Recruitment of the cytoplasmic Ras effector protein Raf kinase *(8)* and the CDC42 Rho-GTPase effector Pak65 *(9)*, to the inner leaflet of the plasma membrane was demonstrated to be sufficient for stimulation of their kinase activity and the activation of the corresponding MAPK signaling cascade.

In my laboratory, we have taken advantage of this general mechanism in signal transduction and the fact that homologous pathways exist in mammalian and yeast cells and developed several approaches on this theme to study protein-protein interactions. The systems are collectively designated "protein recruitment systems" *(10)*. In principle, association between two hybrid proteins results in the translocation of the effector protein to its site of action at the inner leaflet of the plasma membrane. The systems can be studied both in the yeast *Saccharomyces cerevisiae* and in mammalian cells.

1.1. Sos Recruitment System

The Sos recruitment system (SRS) is based on the fact that localization of the human Ras guanyl nucleotide exchange factor, hSos, is sufficient for activation of the Ras signaling pathway. hSos can be directly localized to the plasma membrane by fusion to either v-Src myristoylation signal or Ha-Ras CAAX motive. This regulatory mechanism functions in both mammalian and yeast cells *(5)*. In addition, Sos membrane localization can be achieved via the interaction between two heterologous proteins *(11)*: one that is fused to Sos and the other to the v-Src myristoylation signal. This represents a bona fide system to study interaction between known proteins, as well as to identify of novel protein interactions via a library screening approach (e.g., *see* **refs. 11–15**). Today, multiple cDNA expression libraries compatible with the SRS system are available, which are derived from multiple tissue and cell lines (Stratagene, San-Diego, CA).

1.2. Ras Recruitment System

Following the use of the SRS system, several of pitfalls were identified. First, repetitive isolation of cDNAs encoding for mammalian Ras occurred *(16)*. Second, the expression of multiple baits fused to hSos in Cdc25-2 cells frequently exhibited efficient growth at the restrictive temperature, rendering further analysis of protein–protein interaction impossible *(17)*. Third, the rela-

tively large size of hSos generated problems during bait plasmid construction. To overcome these problems, we have developed the Ras recruitment system (RRS) *(18)*. RRS is based on the absolute requirement for the Ras protein to be localized to the inner leaflet of the plasma membrane for its function *(7)*. The bait of interest is fused to Ras at either the C- or N-terminal. RRS, like SRS, can be used to test known and novel protein–protein interactions *(18,19)*. In addition, RRS is functional not only in yeast, but can also be used in mammalian cells *(20)*. This provides the possibility of verifying and quantitating the protein–protein interaction using Ras-responsive reporter gene assays.

1.3. Reverse Ras Recruitment System

The RRS system cannot be used with baits that encode for either integral membrane proteins or proteins that are associated with the yeast membrane components. To be able to screen for protein–protein interactions with integral membrane proteins, we developed the reverse Ras recruitment approach *(21)*. The system is based on the expression of the native membrane protein with no fusion. The protein is localized to the yeast plasma membrane via its endogenous sequences and, therefore, preserves the proper folding and protein association surfaces. The cDNA encoding for the prey is fused in frame to cytoplasmic Ras at either the C- or N-terminal domain. To eliminate the isolation of clones exhibiting cell growth independent of protein–protein interactions, the dual inducible promoter system was developed. The reverse RRS system can be used to test interactions between known proteins, as well as to identify novel protein–protein interactions following a cDNA library screening protocol.

2. Materials

2.1. Yeast Media

1. Ynb galactose medium (500 mL): 0.85 g of yeast nitrogen without amino acids (0335-15-9; Difco), 2.5 g of ammonium sulfate, 15 g of galactose (G-0750; Sigma, St. Louis, MO), 10 g of D-raffinose (Sigma R-0250), 10 g of glycerol, 20 g of Bacto agar (0140-07-4; Difco).
2. Ynb glucose medium (500 mL): 0.85 g of yeast nitrogen without amino acids, 2.5 g of ammonium sulfate, 10 g of glucose (G8270; Sigma), 20 g of Bacto agar.
3. Add the following amino acids to a final concentration of 50 ng/mL excluding the amino acids that are encoded by the transfected plasmid: leucine (L-8125), uracyl (U-0750), tryptophan (T-0271), lysine (L-5626), adenine (A-3159), histidine (H-9511). Add methionine (M-2893) to a final concentration of 200 ng/mL to achieve efficient repression of the expression derived from the met425 promoter. All amino acids used are from Sigma.
4. YPD medium: 1% yeast extract (0127-17-9; Difco), 2% Bacto peptone (0118-17-0; Difco), 2% glucose, 4% Bacto agar.

2.2. Yeast Solutions

1. LISORB: 100 mM LiAc, 1 M Sorbitol, in TE (10 mM Tris, pH 8.0, 1 mM EDTA).
2. LIPEG: 40% PEG $_{3350,}$ 100 mM LiAc in TE.
3. Salmon sperm DNA: Salmon sperm (D-1626; Sigma). Prepare stock solution at 10 mg/mL and sonicate for 10 min. Prior to transfection, boil salmon sperm for 10 min and cool on ice for 5 min.
4. STET: 8% sucrose, 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 5% Triton X-100.

2.3. Mammalian Cell Transformation Solutions

1. HBS (2X): 50 mM HEPES, 280 mM NaCl, 1.5 mM Na$_2$HPO$_{4,}$ adjust pH to 7.1 with NaOH.
2. Chloramphenicol acetyl transferase (CAT) reaction mixture/reaction: 40 μL of 1 M Tris-HCl, pH 8.0; 3 μL of n-butyryl coenzyme A (5 mg/mL), 1 μL of C^{-14}-chloramphenicol, 6 μL of double distilled water (DDW).

2.4. Other Reagents and Equipment

1. Biodegradable counting scintillant (BCS) (NBCS104; Amersham).
2. n-Butyryl coenzyme A (B-1508; Sigma).
3. C^{-14}-Chloramphenicol (NEC-408; NEN).
4. Dimethylsulfoxide (DMSO) (D-2650; Sigma).
5. Glass beads (425–600 μ) (G-8772; Sigma).
6. HEPES (H-0891; Sigma).
7. Luciferase assay reagent (E1500; Promega).
8. Tetramethylpentadecane (T-7640; Sigma).

3. Methods

3.1. Bait Plasmid Construction

The protein of interest is fused in frame with the c-Myc-epitope tag cytoplasmic-Ras using conventional molecular biology procedures. The yeast expression vector to be used is derived from Met425 vector *(22)*. The plasmid complements leucine auxotrophy and highly expresses the fusion protein when yeast cell transformants harboring the plasmid are grown on medium lacking methionine. In the presence of 200 ng/mL of methionine, the expression levels are reduced to undetectable levels; however, residual expression owing to promoter leakiness is possible. Following plasmid construction, yeast transformants harboring the Ras-Bait expression plasmid are tested for their ability to grow on different media at both restrictive and permissive temperatures to test self-activation and possible toxicity, respectively. In addition, the ability to repress the expression from the met425 promoter by methionine should be tested using Western blot analysis with antibodies generated against the bait protein or against 9E-11 anti-Myc epitope antibody. In the event that

the bait encodes for a membrane protein, the cDNA is designed under the control of the methionine promoter and expressed with no fusion to additional sequences.

3.2. General Yeast Transformation Protocol

The yeast strain used is the *CDC25-2*: *MATα ura3 lys2 leu2 trp1 cdc25-2 his3Δ200 ade101 GAL+*. The following controls should be included in every transfection: Plate ~10^6 cells (100 μL of culture ready for transfection) into a YPD plate incubated directly at 36°C. This control tests the cdc25-2 culture and provides an estimation for the rate of revertants. Typically 10–20 colonies exhibit growth at 36°C. In addition, a control transfection tube should include both pYes2 and pADNS expression plasmids. Following the transfection procedure, plate the control transformants on a Ynb glucose (lacking leucine and uracyl) plate and incubate directly at 36°C. This control gives an estimate regarding revertants and possible contamination accumulated during the transfection procedure. No colony is expected to grow on this plate. For more details, *see* **Note 1**.

1. Place a single cdc25-2 yeast colony into 200 mL of YPD medium. Grow the cells overnight at 24°C to logarithmic phase $2–10 \times 10^6$ cells/mL.
2. Pellet the cells for 5 min at 2500 rpm and resuspend the pellet in 20 mL of LISORB. Following two washes with LISORB, resuspend the cells with LISORB to $2–5 \times 10^8$ cells/mL, and rotate the cells for 30 min at 24°C.
3. For each transfection tube, add 10 μL of preboiled sheared salmon sperm DNA (20 mg/mL) and 2 to 3 μg of each expression plasmid DNA.
4. Mix the DNA by vortexing, and add 200 μL of the prewashed cells from **step 2**. Vortex the cell-DNA mixture briefly, add 1.2 mL of LIPEG, and mix well. Incubate for 30 min at room temperature with constant rotation.
5. To increase transfection efficiency, add 100 μL of DMSO and mix well before a 10-min heat shock at 42°C.
6. Spin the transfection mixture for 1 min and discard the supernatant. Respin for 30 s to completely remove the remaining PEG with a 200-μL tip.
7. Resuspend the pellet in 150 μL of 1 *M* sorbitol, and plate the cells on glucose plates containing the appropriate amino acids and bases. Incubate the plates in a 24°C humidified incubator for 4 d.

3.3. Library Screening for SRS/RRS (see *Fig. 1*)

Library screening with the SRS/RRS systems requires the use of special libraries. The cDNA library is routinely inserted fused to the v-Src myristoylation signal through *Eco*RI-*Xho*I in the pYes2-(URA)-derived expression vector. The plasmid complements the uracyl auxotrophy, and the library prey protein is designed under the control of the GAL1 promoter. The protein

Flow Chart RRS
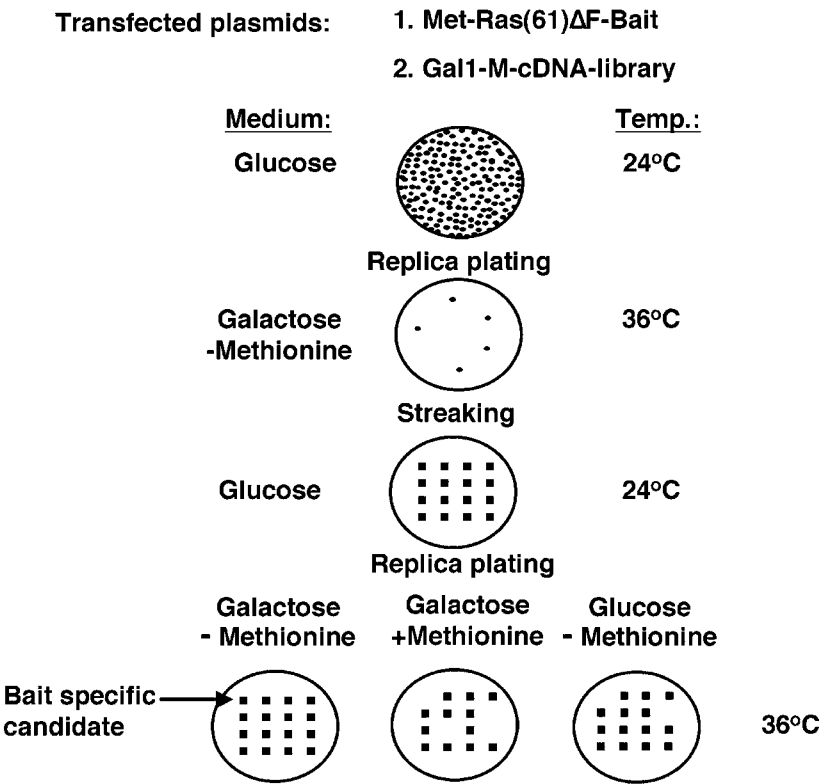
Fig. 1. Flow chart for RRS. Cdc25-2 cells are cotransfected with both Met425-Ras-Bait and Yes-M-cDNA expression plasmids and selected at the permissive temperature on glucose plates containing the appropriate amino acids and bases. Following 5–7 d, plates are replica plated onto galactose-containing plates lacking methionine and incubated at the restrictive temperature. Only a few colonies (10–20) exhibit efficient growth following 3–5 d at the restrictive temperature. Colonies are picked onto a glucose plate containing appropriate bases and amino acids followed by replica plating onto three plates (as indicated) and are incubated at 36°C. Clones that exhibit efficient growth on galactose-containing plates lacking methionine but not on other plates are further analyzed.

is highly expressed when transformants are grown on galactose-containing medium and repressed when grown on glucose. Typically, cotransfection of the bait and library plasmid is performed. The screening flow chart is depicted in **Fig. 1** and described next.

1. The CDC25-2 cells culture overnight (200 mL) at 24°C are used to transform 20 tubes containing the library expression plasmid (3 μg) and the bait expression plasmid (3 μg) resulting in 5000–10,000 transformants on each 10-cm glucose-containing plate lacking leucine and uracyl.

2. Following 5–7 d at 24°C, replica plate the plates onto galactose containing medium (lacking leucine, uracyl, and methionine) and incubated for 3–5 d at 36°C. Select colonies that exhibit growth, place on a glucose plate containing the appropriate amino acids and bases, and incubate at 24°C for 2 d. These clones are tested for their ability to grow at 36°C depending on the expression of both the prey and the bait proteins. Only those clones that exhibit preferential growth when grown on galactose medium lacking methionine at 36°C are considered candidates.

3. To test the specificity of the library plasmid, extract the plasmid DNA from candidate clones and use to cotransform cdc25-2 cells with either the specific bait or a nonrelevant bait. Candidate clones that exhibit bait-specific growth are further analyzed.

3.4. Library Screening for Reverse RRS (see *Fig. 2*)

Library screening for the reverse RRS systems requires the use of special libraries. The cDNA library is inserted fused to the C-terminal of cytoplasmic-Ras through *Eco*RI-*Xho*I in the pYes2-(URA)-derived expression vector. The vector complements the uracyl auxotrophy and highly expresses the prey protein when cells are grown on galactose-containing medium. Transfection of yeast cells is performed as described in **Subheading 3.3.** However, since about 5% of colonies will grow at the restrictive temperature independent of the bait expression, owing to association with the yeast membrane resulting in translocation of Ras to the membrane independent of protein–protein interaction, the library is plated at a low density of about 3000–5000 colonies/10-cm plate. Following 1 wk at 24°C, transformants are replica plated onto two galactose-containing plates either including or lacking methionine and are incubated at 36°C. The pattern of growth of colonies at the restrictive temperature is compared, and only those colonies that exhibit efficient growth in the absence of methionine but not in the presence of methionine are selected for a secondary methionine and galactose-dependent test as described in **Subheading 3.3., step 2.**

3.5. DNA Plasmid Isolation From Yeast

The candidate clone is cultured overnight at 24°C in glucose liquid (3 mL) medium lacking leucine and uracyl.

1. Pellet the cells at 2000 rpm for 5 min and wash once with 1 mL of DDW.

2. Resuspend the pellet in 100 μL of STET and add 0.2 g of 0.45-mm glass beads. Then vigorously vortex for 5 min.

3. Add 100 μL of STET and briefly vortex. Make a hole in the lid of the Eppendorf tube before boiling for 3 min.

Flow Chart Reverse RRS

Transfected plasmids:
1. Gal1-Ras(61)ΔF-cDNA-library
2. Met-membrane-bait

Medium: Temp.:

Glucose   24°C

Replica plating

Galactose   Galactose   36°C
-Methionine   +Methionine

Streaking

Glucose   24°C

Replica plating

Galactose   Galactose   Glucose
- Methionine   +Methionine   -Methionine

Bait specific
candidate   36°C

Fig. 2. Flow chart for reverse RRS. Cdc25-2 cells are cotransfected with both Met425-Bait and Yes-Ras-cDNA expression plasmids and selected at the permissive temperature on glucose plates containing appropriate amino acids and bases. Following 5–7 d, plates are replica plated onto two galactose-containing plates. The first plate lacking methionine and the second plate containing methionine are both incubated at the restrictive temperature. About 150–300 colonies exhibit efficient growth following 3–5 d at the restrictive temperature. Colonies that exhibit growth on the galactose plate lacking methionine but not on the one including methionine are selected onto a glucose plate containing appropriate bases and amino acids followed by replica plating onto three plates (as indicated) and are incubated at 36°C. Clones that exhibit efficient growth on the galactose-containing plate lacking methionine but not on other plates are further analyzed.

4. Cool on ice and spin in a microfuge for 10 min at 4°C.
5. Transfer 100 μL of the supernatant to 50 μL of 7.5 M ammonium acetate, incubate at –20°C for 1 h then centrifuge for 10 min at 4°C.
6. Transfer 100 μL of the supernatant to 200 μL of ice-cold ethanol. Mix well and recover the DNA by centrifuging for 10 min at 4°C.
7. Wash with 150 μL of 70% ethanol, and resuspend the dried pellet in 20 μL of DDW. For more considerations, *see* **Notes 2** and **3**.

3.6. 293-HEK Transfection

Following identification and verification of a DNA plasmid that provides efficient yeast growth at the restrictive temperature only in the presence of the specific bait, it is possible to test the interaction directly in mammalian cells. To this end, 293-human embryonic kidney cells (300,000) are plated on 60-mm plates 1 d before transfection. Twelve micrograms of high-quality PEG DNA plasmid preparation is used for each transfection. The DNA mixture contains 3 μg of each of the following plasmids: Polyoma enhancer-CAT reporter gene, 4XAP-1-luciferase reporter gene, pcDNA (Invitrogen)-derived bait expression plasmid, and prey expression plasmid. In control transfection in which either the bait or prey expression plasmids are omitted, pcDNA empty expression vector is used to adjust the DNA content, respectively. The volume of DNA mixture is adjusted to 450 μL with sterile DDW, and 50 μL of 2.5 M $CaCl_2$ is added. The DNA-$CaCl_2$ mixture is slowly added into a sterile tube containing 500 μL of 2X HBS by air bubbling and incubated for 15 min at room temperature. Five hundred microliters of the transfection mixture is added to the cells. Following 5 h, the medium is replaced with fresh medium. Cells are harvested 40 h following addition of the DNA to the cells. Cells are collected by resuspending in 1 mL of phosphate-buffered saline followed by centrifugation. The cell pellet is resuspended in 100 μL of 100 mM potassium phosphate buffer, pH 7.8, containing 1 mM dithiothreitol. Cell extract is prepared by three cycles of freeze (liquid nitrogen) and thaw (37°C) followed by 5 min of centrifugation at 4°C. The supernatant is transferred to new tubes and used for further analysis.

3.7. Luciferase Reporter Assay

A luciferase assay is performed with 10–25 μL of cell extract using the luciferase assay system (Promega) according to the manufacturer's instructions, measured by a TD-20/20 luminometer (Turner Designs).

3.8. CAT Reporter Assay

1. Perform the CAT assay with 10–25 μL of cell extract adjusted to 50 μL with DDW.
2. Add 50 μL of CAT reaction mixture and incubate at 37°C for 1 h.
3. Stop the enzymatic reaction by adding 200 μL of TMPD/Xylene (2:1) and vortexing for 1 min followed by 2 min of centrifugation at 16,000g.
4. Transfer 100 μL of the acetylated upper phase into scintillation tubes containing 1 mL of BCS.
5. Determine the percentage of the acetylated chloramphenicol using a conventional β-counter.

4. Notes

1. Transformants incubated at 24°C appear 3 to 4 d following transfection. Single colonies are plated using a grid plate on Ynb glucose (lacking leucine and uracyl) grown at 24°C for two additional days before further replica plating into both Ynb galactose and Ynb glucose containing appropriate bases and amino acids and YPD plates incubated at 36°C.

2. In the event that the library plasmid provides a selection marker identical to the bait, an optional digestion step can be performed in order to increase the yield of library plasmid isolation. The DNA mixture can be digested with a rare restriction enzyme, *Not*I, which linearizes the bait but has no recognition site within the library-derived expression plasmid. Following 1 h of digestion, DNA is extracted by phenol/chloroform and recovered by ethanol precipitation using 2 μL of tRNA (10 mg/mL) as carrier. The DNA is dissolved in 10 μL of DDW and used to transform the bacteria.

3. In principle, yeast candidate clones contain two different DNA plasmids (bait and prey expression plasmids). Commercially available expression plasmid libraries compatible with the SRS and RRS (Stratagene) were designed to provide chloramphenicol resistance and therefore, the isolation of the library plasmid is highly facilitated. Highly competent bacteria are used to transform 1 μL of the isolated plasmid DNA. Bacteria are plated on Luria Burtani + amplification (100 μg/mL), and single colonies are selected for the preparation of single DNA miniprep to be further analyzed by digestion with *Eco*RI-*Xho*I restriction enzymes for identification of the cDNA inserts. Individual library plasmids are used to retransform cdc25-2 yeast cells with either the specific bait or a nonspecific bait.

References

1. Chang, L. and Karin, M. (2001) Mammalian MAP kinase signalling cascades. *Nature* **410,** 37–40.
2. Carpenter, G. (2000) The EGF receptor: a nexus for trafficking and signaling. *Bioessays* **22,** 697–707.
3. Huang, D. C. S., Marshall, C. J., and Hancock, J. F. (1993) Plasma membrane targeted ras GTPase-activating protein is a potent suppressor of p21ras function. *Mol. Cell. Biol.* **13,** 2420–2431.
4. Klippel, A., Reinhard, C., kavanaugh, M., Apell, G., Escobedo, M. A., and Williams, L. T. (1996) Membrane localization of phosphatidylinositol 3-kinase is sufficient to activate multiple signal-transducing kinase pathways. *Mol. Cell. Biol.* **16,** 4117–4127.
5. Aronheim, A., Engelberg, D., Li, N., Al-Alawi, N., Schlessinger, J., and Karin, M. (1994) Membrane targeting of the nucleotide exchange factor Sos is sufficient for activating the Ras signaling pathway. *Cell* **78,** 949–961.
6. Quilliam, L. A., Huff, S. Y., Rabun, K. M., Wei, W., Park, W., Broek, D., and Der, C. J. (1994) Membrane-targeting potentiates guanine nucleotide exchange factor CDC25 and SOS1 activation of Ras transforming activity. *Proc. Natl. Acad. Sci. USA* **91,** 8512–8516.

7. Hancock, J. F., Magee, A. I., Childs, J., and Marshall, C. J. (1989) All ras proteins are polyisoprenylated but only some are palmitoylated. *Cell* **57,** 1167–1177.

8. Leevers, S. J., Paterson, H. F., and Marshall, C. J. (1994) Requirement for ras in Raf activation is overcome by targeting Raf to the plasma membrane. *Nature* **369,** 411–414.

9. Lu, W., Katz, S., Gupta, R., and Mayer, B. J. (1997) Activation of Pak by membrane localization mediated by an SH3 domain from the adaptor protein Nck. *Curr. Biol.* **7,** 85–94.

10. Aronheim, A. (2000) Protein recruitment systems for the analysis of protein-protein interactions. *Biochem. Pharmacol.* **60,** 1009–1013.

11. Aronheim, A., Zandi, E., Hennemann, H., Elledge, S., and Karin, M. (1997) Isolation of an AP-1 repressor by a novel method for detecting protein-protein interactions. *Mol. Cell. Biol.* **17,** 3094–3102.

12. Yu, X., Wu, L. C., Bowcock, A. M., Aronheim, A., and Baer, R. (1998) The C-terminal (BRCT) domains of BRCA1 interact in vivo with CtIP, a protein implicated in the CtBP pathway of transcriptional repression. *J. Biol. Chem.* **273,** 25,388–25,392.

13. Andreev, J., Simon, J. P., Sabatini, D. D., Kam, J., Plowman, G., Randazzo, P. A., and Schlessinger, J. (1999) Identification of a new Pyk2 target protein with Arf-GAP activity. *Mol Cell Biol.* **19,** 2338–2350.

14. Ko, L., Cardona, G. R., and Chin, W. W. (2000) Thyroid hormone receptor-binding protein, an LXXLL motif-containing protein, functions as a general coactivator. *Proc Natl Acad Sci USA* **97,** 6212–6217.

15. Barak, O., Aronheim, A., and Shaul, Y. (2001) HBV X protein targets HIV Tat-binding protein 1. *Virology* **283,** 110–120.

16. Aronheim, A. (1997) Improved efficiency Sos recruitment system: expression of the mammalian GAP reduces isolation of Ras GTPase false positives. *Nucleic Acids Res.* **25,** 3373,3374.

17. Aronheim, A. (2001) Protein recruitment systems for the analysis of protein-protein interactions. *Methods* **24,** 29–34.

18. Broder, Y. C., Katz, S., and Aronheim, A. (1998) The Ras recruitment system, a novel approach to the study of protein-protein interactions. *Curr. Biol.* **8,** 1121–1124.

19. Aronheim, A., Broder, Y. C., Cohen, A., Fritsch, A., Belisle, B., and Abo, A. (1998) Chp, a homologue of the GTPase Cdc42, activates the JNK pathway and is implicated in reorganizing the actin cytoskeleton. *Curr. Biol.* **8,** 1125–1128.

20. Maroun, M. and Aronheim, A. (1999) *A novel in vivo assay for the analysis of protein-protein interaction. Nucleic Acids Res.* **27,** e4.

21. Hubsman, M. Y. G., and Aronheim, A. (2001) A novel approach for the identification of protein-protein interaction with integral membrane proteins. *Nucleic Acids Res.* **29,** e18.

22. Mumberg, D., Muller, R., and Funk, M. (1994) Regulatable promoters of Saccharomyces cerevisiae: comparison of transcriptional activity and their use for heterologous expression. *Nucleic Acids Res.* **22,** 5767–5768.

18

Methods in Functional Proteomics

*Two-Dimensional Polyacrylamide Gel Electrophoresis
With Immobilized pH Gradients, In-Gel Digestion,
and Identification of Proteins by Mass Spectrometry*

**Karine R. Bernard, Karen R. Jonscher, Katheryn A. Resing,
and Natalie G. Ahn**

1. Introduction

The recent combination of several technology platforms has revolutionized the analysis of expressed cellular proteins (proteomics). Large-scale separation and visualization of proteins is accomplished using two-dimensional gel electrophoresis (2-DE). Proteins are separated first by isoelectric focusing (IEF) using immobilized pH gradient (IPG) strips. Following charge separation, proteins are further resolved by molecular mass using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as shown in **Fig. 1**.

Image analysis of stained 2-D gels from cells under various states of stimulation or disease can be used to screen proteins that are differentially expressed (functional proteomics). Identifying and characterizing the structure/function of these proteins may lead to possible drug targets. Large-scale, automated protein identification can be accomplished using mass spectrometry (MS) combined with powerful database searching algorithms. Since an initial study on the yeast proteome by Shevchenko et al. *(1)* in 1996, functional proteomics utilizing 2-DE separation and MS has been reported in many applications, including identification of proteins involved in signaling pathways *(2,3)*, as well as in cancer *(4–6)*.

Dried gel strips containing IPG were first described in 1982 *(7,8)* and commercially introduced in 1991. IPG strips are manufactured by copolymerizing acidic and basic acrylamido derivatives of different pKs within a polyacrylamide matrix. The covalent nature of the chemical bonds formed during the
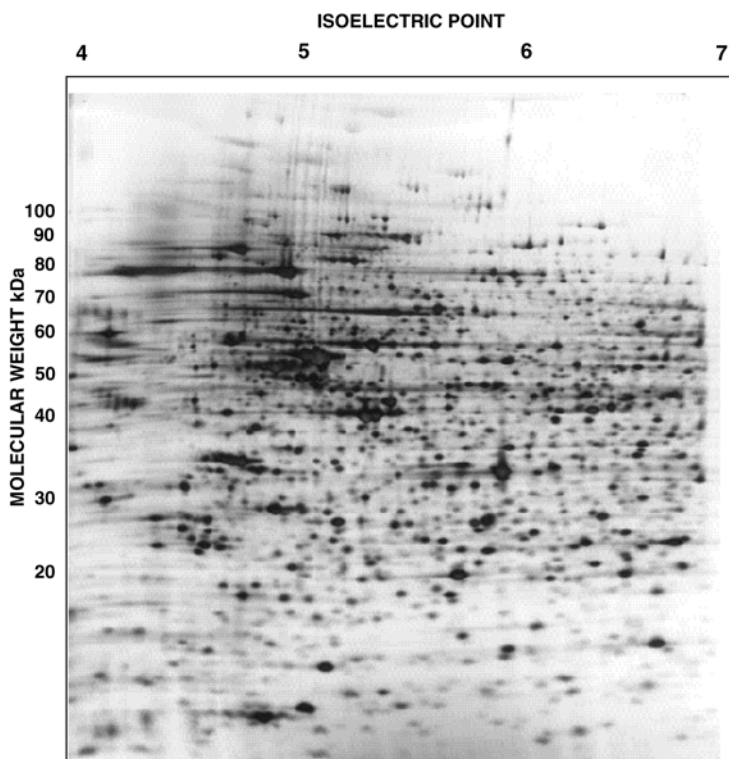
ISOELECTRIC POINT

Fig. 1. 2-DE gel pattern of WM35 (melanoma) cell line.

polymerization step results in permanent stability of the pH gradient within the matrix with a typical pI resolution of 0.01 pH units. The acrylamide matrix with the acrylamido buffers is cast onto a Gelbond backing sheet, polymerized, washed, and dried. The backing stabilizes the size of the strips and simplifies handling.

Samples are applied to the IPG strips by rehydration. The rehydrated strips are then placed onto the cooling plate of an electrofocusing chamber for separation in the first dimension. Prior to the second-dimension run, IPG strips are equilibrated in solutions in order to reduce and alkylate cysteine. Following separation by mass in the second dimension, various stains are employed to detect proteins *(9)*. Computerized image analysis software is used to select spots of interest, which are subsequently digested with proteases to produce peptides that are analyzed using MS. Accurately measured masses of four to six peptides can be used to unambiguously identify a protein by searching databases such as the nonredundant protein databases housed at NCBI *(10)*. The

"peptide mass mapping" technique is typically performed using a time-of-flight (TOF) mass spectrometer.

In a complementary approach, peptides in digests can be fragmented into sequence-specific product ions using tandem MS. The product ion spectrum is compared with computer-generated spectra derived from protein sequences in databases for protein identification. If complete sequence information is not available, a partial sequence tag can be deduced and used to search for homologous proteins containing the tag. The combination of a single peptide sequence and molecular weight may be sufficient to unambiguously identify a protein, provided that the protein of interest is represented in the sequence database.

This review outlines methods for resolving whole-cell mammalian extracts by high-resolution 2-DE and subsequent in-gel protein digestion. Methods for analyzing digests by MS depend on the instruments used and are not covered in detail. However, practical concepts important for successful peptide mapping and sequencing are included.

2. Materials

2.1. Sample Preparation

1. Lysis buffer components recommended for mammalian cell extractions are listed in **Table 1**. CHAPS and thiourea are from Sigma (St. Louis, MO). Dithiothreitol (DTT) and ultrapure electrophoresis-grade urea are from Fisher (Pittsburgh, PA), and IPG buffer (pH 4.0–7.0) is from Amersham Pharmacia Biotech (Uppsala, Sweden). Ideally, fresh lysis buffer should be used to lyse the cells. However, the buffer can also be stored in aliquots (1 mL) at –80°C, in which case DTT should be added immediately before cell lysis.
2. D-PBS: Dulbecco's phosphate-buffered saline (containing no calcium or magnesium). Powder is from Gibco-BRL (Rockville, MD).
3. TLA100 tabletop ultracentrifuge for clarifying lysates. Ultracentifuge, TLA100.2 rotor, and polypropylene TLA100.2 centrifuge tubes are from Beckman Instruments.
4. Bradford Protein Assay reagent, used to determine protein concentration, is from Bio-Rad (Richmond, CA). Bovine serum albumin standard solution (2 mg/mL) is from Pierce.

2.2. Rehydration of IPG Strips With Sample

1. Lysis buffer and rehydration buffer are described, respectively, in **Tables 1** and **2**.
2. Immobiline DryStrip reswelling tray (Amersham Pharmacia Biotech).
3. Immobiline DryStrips (pH 4.0–7.0), 18 cm (Amersham Pharmacia Biotech).
4. Light mineral oil (paraffin oil, light) and forceps (fine tipped) (Fisher).
5. High-performance blot paper (46 cm × 57 cm × 3 mm) (Life Science, Denver, CO). This is similar in weight to Whatman 3MM filter paper.

Table 1
Lysis Buffer Components

Buffer component (final concentration)	Stock solution	Final vol = 10 mL
7 *M* Urea	Powder	4.2 g
2 *M* Thiourea	Powder	1.52 g
4% (w/v) CHAPS	Powder	0.4 g
1 m*M* Benzamidine	1 *M*	10 µL
25 µg/mL Leupeptin	20 mg/mL in water	12 µL
20 µg/mL Pepstatin	10 mg/mL in ethanol	20 µL
10 µg/mL Aprotinin	50 mg/mL in 10 m*M* HEPES, pH 7.4	2 µL
1 m*M* DTT	1 *M* DTT in water (prepared fresh)	10 µL
1 m*M* Sodium orthovanadate	1 *M*	10 µL
1 m*M* Microcystin	1 m*M* in dimethylsulfoxide	10 µL
1% (v/v) Ampholines	100% IPG buffer, p*I* 4–7	100 µL
Deionized water (MilliQ)		Up to 10 mL

Table 2
Rehydration Buffer

Buffer component	Stock solution	Final vol = 10 mL
7 *M* Urea	Powder	4.2 g
2 *M* Thiourea	Powder	1.52 g
4% (w/v) CHAPS	Powder	0.4 g
50 m*M* DTT	1 *M* DTT	500 µL
3% (v/v) Ampholines	100% IPG buffer	300 µL
Bromophenol blue	10% (w/v) in water	20 µL
Deionized water (Milli Q)		Up to 10 mL

2.3. First Dimension: IEF

1. Multiphor II flatbed electrophoresis unit, electrode holder, Immobiline strip aligner, cathode electrode, anode electrode, and IEF electrode strips (Amersham Pharmacia Biotech).
2. Programmable electrophoresis power supply (EPS 3500 XL) (Amersham Pharmacia Biotech).
3. The power supply gradient program for electrophoresis of Immobiline DryStrips (pH 4.0–7.0) (for description, *see* **Table 3**).
4. Light mineral oil (Fisher).

Table 3
Program for Electrophoresis of Immobiline DryStrip pH 4.0–7.0, 180 mm Long

Phase	Voltage (V)	Current (mA)	Power (W)	Time (h)	V-h
1	300	2	7	0.01	3
2	300	2	7	1	300
3	3500	2	7	9	31,500
4	3500	2	7	18	63,000
Total				28	94,803

Table 4
Equilibration Solution

Equilibration solution component	Stock solution	Final vol = 200 mL
6 M Urea	Powder	72 g
0.05 M Tris-HCl (pH 6.8)	0.5 M	20 mL
30% (v/v) Glycerol	100%	60 mL
2% (w/v) SDS	Powder	4 g
Deionized water (MilliQ)		Up to 200 mL

2.4. Reduction and Alkylation

1. Glass screw-cap test tubes (200-mm long, 20-mm id, 70-mL capacity) (Fisher).
2. Components of the reduction and alkylation solution (for description, *see* **Table 4**).

2.5. Second Dimension: SDS-PAGE

1. Sandwich gels were cast using Protean II xi plate (20 × 20 cm) sets, including an outer, inner, and notched middle plate with four spacers. Plates, spacers, sandwich clamps, and casting stands (Bio-Rad).
2. 30% Acrylamide/0.8% bisacrylamide, 37.5:1 (2.6°C) solution (Bio-Rad).
3. Ammonium persulfate (APS) (Sigma).
4. *N,N,N',N'*-tetramethylethylenediamine (TEMED) (Boehringer Mannheim, Mannheim, Germany).
5. Two liters of 1X running buffer, containing 28.8 g of glycine (final: 60 mM), 6.06 g of Tris base (final: 25 mM), and 20 mL of 10% SDS (final: 0.1%).
6. Glycine and 1-butanol (Fisher).
7. Ultra pure agarose (Gibco-BRL). A solution of 0.5% (w/v) agarose is made by dissolving 0.25 g of agarose in 50 mL of running buffer, heated to 100°C in a water bath.
8. Protean II xi cell (tank, lid, and central cooling core), to run gels (Bio-Rad).
9. Refrigerated water circulator (Model 1160) (VWR, West Chester, PA).

10. For nongradient gels: Four gels require 150 mL of a 12% acrylamide/0.32% bisacrylamide solution, containing 60 mL of 30% acrylamide/bisacrylamide (37.5:1) solution, 37.5 mL of 1.5 M Tris-HCl (pH 8.8), 500 μL of 10% APS, 50 μL of TEMED, and 52 mL of deionized water.
11. For 8–18% gradient gels:
    a. Four gels require 80 mL of an 8% acrylamide/0.21% bisacrylamide (low acrylamide) solution containing 21.3 mL of 30% acrylamide/bisacrylamide (37.5:1) solution, 20 mL of 1.5 M Tris-HCl (pH 8.8), 260 μL of 10% APS, 26 μL of TEMED, and 38.5 mL of deionized water.
    b. Four gels require 80 mL of an 18% acrylamide/0.48% bisacrylamide (high acrylamide) solution containing 48 mL of 30% acrylamide/bisacrylamide (37.5:1) solution, 20 mL of 1.5 M Tris-HCl (pH 8.8), 260 μL of 10% APS, 26 μL of TEMED, and 11.7 mL of deionized water.
12. Gradient maker (Hoefer Model SG30; Amersham Pharmacia Biotech) and peristaltic pump (Model RP-I; Rainin, Woburn, MA). A standard power supply for electrophoresis can be used in the second dimension.

2.6. Staining 2-DE Gels

2.6.1. Silver Staining

1. 37% (w/v) Formaldehyde solution (Fisher).
2. Silver nitrate, 99+% (Sigma).

2.6.2. Coomassie Staining

1. Coomassie staining solution: 25% isopropanol, 10% acetic acid, and 0.05% Coomassie Brilliant Blue R-250 (USB, Cleveland, OH).
2. Coomassie destaining solution: 10% acetic acid and 10% methanol in water.
3. Cellophane supports, drying frames, and air-drying chamber (Bio-Rad).

2.7. In-Gel Digestion of Proteins Separated by 2-DE

1. Ammonium bicarbonate (NH_4HCO_3) and potassium ferricyanide (Sigma).
2. Water and acetonitrile (high-performance liquid chromatography [HPLC] grade) (Fisher).
3. Sequencing grade, modified porcine trypsin (Promega; Madison, WI).
4. Formic acid (88%) (Mallinckrodt-Baker)
5. ZipTip-C18 reverse-phase columns, for desalting (Millipore, Bedford, MA).

2.8. Peptide Mapping and Sequencing by MS

1. Matrix, α-cyano-4-hydroxy-*trans*-cinnamic acid (Hewlett Packard, Santa Clara, CA).
2. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer, Voyager-DE STR, and stainless steel 100-well sample plates (PE Applied Biosystems, Foster City, CA).
3. LCQ Classic ion trap mass spectrometer (ThermoFinnigan, San Jose, CA).
4. PicoView nanospray source and PicoFrit columns (New Objective, Woburn, MA).

5. Magic 2002 HPLC pump (Michrom Biosources, Auburn, CA).
6. Hamilton Gastight 1700 series syringes (Fisher).
7. PEEK tubing (1/16" o.d. and 360 μm o.d.) (Upchurch, Whidbey Island, WA).

3. Methods

3.1. Sample Preparation

Sample preparation is the most crucial step for successfully separating proteins by 2-DE. Noncovalent interactions, such as ionic bonds, hydrogen bonds, hydrophobic interactions, and disulfide bridges, must be disrupted under conditions compatible with IEF. Only very low amounts of salts or other charged compounds may be present in the solvent.

Chaotropes dramatically decrease ionic interactions by altering the dielectric constant of the solvent and by denaturing proteins. For 2-DE, the chaotrope of choice for whole cell lysates is a mix of urea and thiourea (*see* **Note 1**). A detergent such as CHAPS is included in the lysis buffer mixture to improve solubility.

The following protocol has been optimized for mammalian cells, growing adherent or in suspension:

1. Wash 5×10^6 cells with 10 mL of D-PBS twice. Drain D-PBS thoroughly by aspiration without letting the cells go dry.
2. Place a minimum amount of lysis buffer onto the cells. For example, add 700 μL of lysis buffer to adherent cells in a 75-cm^2 tissue culture flask, or add 0.5 mL of lysis buffer to a 5×10^6 suspension of cells, collected by centrifugation. Let the cells incubate in lysis buffer for 5 min, and then collect by scraping and transfer the lysate to a 1.5-mL microcentrifuge tube.
3. Incubate the lysates for 1 h at room temperature to disrupt the cells and precipitate the DNA. Store the lysates at –80°C after this step or after step 4.
4. Clarify the cell lysates by centrifuging at 200,000*g* (22°C) for 1 h to remove insoluble material. This can be done in a Beckman TLA 100 ultracentrifuge using a TLA100.2 rotor at 200,000*g*.
5. Determine the protein concentration of soluble lysate fractions. Various colorimetric protein reagents, such as Bradford Coomassie G250, may be used, but protein standards should be diluted in lysis buffer to correct for background signal.

3.2. Rehydration of IPG Strip With Sample

1. Using measured protein concentrations, calculate sample volumes containing 100–150 mg of total protein. Remove the appropriate volume to a 1.5-mL microcentrifuge tube. For 18-cm DryStrips, add additional lysis buffer to the sample to provide a final volume of 175 μL. Mix and then add 175 μL of rehydration buffer (after the addition of 20 m*M* DTT) to a total volume of 350 μL (when using 18-cm IPG DryStrips).
2. Incubate the mixture at room temperature for 20 min.

3. Level the reswelling tray so that the strips lie horizontally.
4. Pipet each sample solution (350 μL) into a slot in the reswelling tray, being careful to minimize bubbles.
5. Remove the IPG strips from storage at –20°C. Label the backing strip with an ink pen.
6. Remove the protective plastic from the IGP strips and gently place each strip, with the gel side facing down, on top of the solution in each reswelling slot. To eliminate bubbles between the sample and strip, repeatedly raise one half of the strip and reapply it to the solution, and then raise and reapply the other half.
7. Immediately overlay the IPG strip with 2 mL of light mineral oil to prevent evaporation and urea crystallization.
8. Repeat **steps 4–7** for each sample.
9. Slide the lid onto the tray and allow the gel strips to rehydrate with sample overnight (16 h) at 25°C.

3.3. First Dimension: IEF

It is important to perform **steps 4–10** quickly, in order to prevent urea crystallization.

1. Connect the Multiphor II flatbed electrophoresis unit to the cooling water circulator and set to 20°C. Make sure the unit is level.
2. Spread 10 mL of light mineral oil over the Multiphor cooling plate and place the electrode holder on the oil. This permits good contact with the cooling plate and enables even cooling.
3. Spread 10 mL of light mineral oil inside the electrode holder and place a flexible plastic IPG strip aligner (with the 12 grooves concave side up) on the oil. To avoid uneven cooling, ensure that bubbles are not trapped under the plastic strip aligner.
4. Cut two IEF electrode strips to a length of 11 cm. Place the IEF electrode strips onto a piece of blot paper and soak each one with 1 mL of distilled water. Remove excess water by blotting with Kimwipe tissues.
5. Remove the rehydrated IPG strips from the reswelling tray, place them gel side up onto blot paper (6" × 10"), and briefly lay another piece of dry blot paper on top of the gel strips to absorb excess solution.
6. Place the IPG strips gel side up between adjacent concave grooves in the plastic strip aligner. The pointed ends of the strip (low p*I* end) should face the anode (red). After all IPG strips are in the tray, align the basic/cathode side of the gels.
7. Position the moistened IEF electrode strips orthogonally to the tops of the aligned IPG strips, one at each end. The electrode strips act as wicks for ionic compounds.
8. Position the electrodes onto the IEF electrode strips with the platinum wires centered on each strip. Adjust so that the electrodes are level.
9. Flood the electrophoresis tray with light mineral oil (~100 mL) until the IPG strips are completely covered.
10. Set the parameters on the power supply as described in **Table 3**. Running conditions depend on the pH gradient and the length of the IPG gel strip used and

typically include low current and limiting voltage. Optimization of time and gradient is recommended for different sample types.

11. At the end of the run, disconnect the power supply, remove the IPG strips, and gently blot off the excess oil using blot paper.
12. After the first dimension is completed, the IPG strips may be packaged in plastic wrap and stored at –80°C. If the second dimension gel is to be run immediately following IEF, prepare solutions for reduction and alkylation beforehand.

3.4. Reduction and Alkylation

IPG strips are equilibrated in the presence of glycerol, urea, SDS, DTT, and iodoacetamide in order to resolubilize, reduce, and alkylate proteins. The urea and the SDS in the equilibration solution increase the solubility of the proteins and the glycerol minimizes diffusion. DTT is added to reduce disulfide bonds. Iodoacetamide is added to remove excess DTT and alkylate sulfhydryl residues.

1. Prepare 200 mL of equilibration solution for four IPG strips (**Table 4**). Divide the solution into 2 × 100 mL for the following solutions:
   a. Reducing solution: Add 1 g of DTT to 100 mL of equilibration solution.
   b. Alkylating solution: Add 4 g of iodoacetamide and 20 μL of 10% (w/v) bromophenol blue to 100 mL of equilibration solution.
2. Remove the IPG strips from storage at –80°C or from the IEF apparatus. Place each IPG strip individually into a glass screw-cap test tube containing 25 mL of reducing solution, and rock on an orbital shaker at 100 rpm for 15 min.
3. Transfer the IPG strips directly into glass test tubes containing 25 mL of alkylating solution and rock as in **step 2** for 15 min.
4. Following alkylation, place the IPG strips (slightly bent) on one edge of a piece of blot paper (6" × 10"). Immediately proceed to **Subheading 3.5.2., step 1**.

3.5. Second Dimension: SDS-PAGE

During separation by SDS-PAGE, negatively charged SDS is used to coat the protein and modify the intrinsic protein charge. Proteins in a mixture have the same net charge per unit length; thus, movement through the gel depends primarily on the molecular mass of the protein. SDS used in the gel-running buffer is sufficient to maintain a net negative charge on proteins and does not need to be included during polymerization of the vertical SDS gel.

3.5.1. Casting Vertical SDS Gels

1. Assemble sandwich gels carefully to prevent leaking. Each sandwich of two gels consists of one outer plate, one notched plate, one inner plate, and four 1.0 mm spacers in between, assembled between two sandwich clamps (*see* **Note 2**). Small squares of wet filter paper can be used as labels and placed at the bottom corners between the glass plates before casting.

2. Set the sandwich gel into a casting stand.

3. For nongradient gels, pour a 12% acrylamide solution (*see* **Subheading 2.5., item 10**) between the glass plates until the liquid level is 0.5 cm from the top of the shortest glass plate. Proceed to **step 8**.

4. For 8–18% gradient gels, prepare the 8 and 18% acrylamide solutions without TEMED and APS. Chill the solutions on ice for 20 min. Then add TEMED to each solution.

5. Pour the gel acrylamide solutions into the gradient maker attached to a peristaltic pump. Place 18 mL of the 8% solution into the reservoir chamber and 18 mL of the 18% solution into the mixing chamber with a small stir bar. Add APS to each solution, turn on the magnetic stirrer in the mixing chamber, and use a pipet to mix APS in the low-acrylamide solution in the reservoir chamber (*see* **Note 3**).

6. Run the peristaltic pump at 48 mL/min. Allow the tubing to fill with the 18% solution and then open the valve between the mixing and reservoir chambers. Stop the pump when the liquid level reaches 0.5 cm from the top of the glass plates. Ideally, this will coincide with the end of the gradient volume.

7. Repeat **steps 5** and **6** for each gradient gel.

8. Overlay the gels with water-saturated 1-butanol and allow them to polymerize for 4 h at room temperature. Replace the butanol with 375 mM Tris (pH 8.8) if the gels are stored at 4°C. Decant and rinse with deionized water immediately prior to running the second-dimension gel.

3.5.2. Running Second-Dimension Gel

1. Lay an IPG strip on top of an SDS-PAGE gel. The direction is not critical, but consistency is recommended. It might be necessary to cut the plastic edges of the IPG strip at both ends in order for the strip to fit between the spacers.

2. Immediately overlay the IPG strip with 0.5% agarose in 1X running buffer and allow the agarose to solidify. Repeat **steps 1** and **2** for each IPG strip (total of four). Mount two sandwich gels onto the central cooling unit and place in the electrophoresis tank.

3. Fill the electrophoresis tank by adding 1.7 L of 1X running buffer to the bottom of the tank, and 0.3 L of 1X running buffer on top of the gels. Connect the central unit to the cooling water circulator set to 10°C, and then connect the lid to the power supply. Start the electrophoresis at 7 mA/gel for 1 h, and then increase the current to 25 mA/gel.

4. Terminate the run when the Bromophenol blue tracking dye reaches the very bottom of the gel (approx 5 h total).

5. When the run is complete, remove the gel sandwiches from the apparatus and carefully open the glass plates using a plastic spatula.

6. Dispose of the agarose overlay from each polyacrylamide gel, and place each gel into 250 mL of fixing solution for silver staining (*see* **Tables 5** and **6**), or proceed to Coomassie staining. Incubate the gels in fixing solution overnight with gentle shaking. Fixing is required to immobilize the proteins in the gel and to remove nonprotein components such as glycine, Tris, SDS, and carrier ampholytes present at the bottom of the gels, which might interfere with subsequent staining.

Table 5
Silver Staining Protocol for Analytical Gels *(12)*

Step	Reagent (250 mL/gel unless noted)	Duration
Fixation	40% Ethanol/12% acetic acid/0.0185% formaldehyde	Overnight
Wash 1	50% Ethanol	10 min
Wash 2	30% Ethanol	10 min
Sensitization	0.02% Sodium thiosulfate	1 min
Wash	Deionized water	3×20 s
Impregnation	0.2% Silver nitrate/0.075% formaldehyde (125 mL/gel)	30 min
Wash	Deionized water	2×10 s
Development	6% Sodium carbonate/0.0004% sodium thiosulfate/ 0.0185% formaldehyde	2–4 min
Stop	40% Ethanol/12% acetic acid	5 min
Wash	Deionized water	2×10 min

Table 6
Silver Staining Protocol for MS *(13)*

Step	Reagent (250 mL/gel unless noted)	Duration
Fixation	50% Methanol, 5% acetic acid	20 min
Wash 1	50% Methanol	10 min
Wash 2	Deionized water	10 min
Sensitization	0.02% Sodium thiosulfate	1 min
Wash	Deionized water	2×1 min
Impregnation	0.1% Silver nitrate (125 mL/gel)	20 min
Wash	Deionized water	2×1 min
Development	2% Sodium carbonate/0.0148% formaldehyde (500 mL/gel); keep solution transparent, changing solution when it turns yellow	5–8 min
Stop	5% Acetic acid	5 min
Wash	Deionized water	2×10 min

7. After staining (*see* **Subheading 3.6.**), protein spots can be excised immediately or gels can be dried for storage and archiving. Drying gels between cellophane supports facilitates later protein recovery and is preferred to drying gels onto filter paper. To dry gels between cellophane, wet two cellophane supports (32 × 32 cm) with deionized water and lay one on top of a square drying frame (25 × 25 cm) over a clean, flat surface. Place the gel onto the cellophane and remove any bubbles. Overlay the second cellophane support on top of the gel and remove bubbles. Place a second drying frame on top of the second support, and clamp together with the first drying frame on all four sides. The gel can be dried in any clean area or inside an air dryer overnight without heat.

3.6. Staining 2-DE Gels

Proteins separated by 2-DE can be visualized using Coomassie Brilliant Blue, silver staining, imidazole reverse staining *(11)*, or Sypro Ruby fluorescence staining *(9)*. Silver staining is one of the most sensitive methods and was first introduced in 1979 by Switzer et al. *(12)*.

Silver staining can be carried out as described by Blum et al. *(13)*, yielding high-sensitivity analytical gels optimal for image analysis (*see* **Table 5**). However, recovery by in-gel digestion is poor, possibly owing to formaldehyde in the fixation solutions. Improved recovery from in-gel digestion for MS has been observed using the low fixation protocol from Shevchenko et al. *(14)* (*see* **Table 6**), or with some commercial silver staining kits (e.g., SilverQuest, Invitrogen). Both silver stain methods are extremely sensitive and background reduction, with concomitant sensitivity enhancement, is achieved by pretreating the gel with sodium thiosulfate, a reducing agent, prior to impregnating with silver. Gels are impregnated in each solution and rotated on an orbital shaker at 50 rpm for the times indicated in **Tables 5** or **6**. Then each solution is decanted and discarded before adding the next one. For best results, always wear clean gloves and use a plastic sheet between the gel and gloves to decant solutions. Use manual shaking during development (*see* **Notes 4** and **5**). After staining, gels are washed with MilliQ water and preserved by air-drying between cellophane supports (*see* **Subheading 3.5.2., step 7**), or processed immediately for spot cutting.

Coomassie Brilliant Blue staining is less sensitive than silver staining but allows nearly complete recovery of peptides after in-gel digestion. Gels are incubated in Coomassie stain solution for 1 to 2 h, washed two to three times with deionized water, and incubated in destaining solution for 2 to 3 h. Kimwipes can be added to the destaining solution to absorb excess stain.

Sypro Ruby fluorescence stain (Molecular Probes) is about fivefold less sensitive than high-fixation silver staining but has the advantages of greater linearity in intensity facilitating quantitation, and good recovery for MS.

3.7. Image Analysis

Several computerized programs are currently available for image analysis of 2-D gels, including Melanie 4 from GeneBio (Geneva, Switzerland); PD-QUEST distributed by Bio-Rad; Z3 from Compugen; Progenesis™, a Nonlinear Dynamics image analysis software supplied by Amersham Pharmacia Biotech; AlphaMatch™ from Alpha Innotech; and Phoretix 2-D from Nonlinear Dynamics (Durham, NC). Accurate, automated image analysis that can be used for high-throughput proteomics applications is still difficult using software currently available, although the programs are rapidly evolving toward

this goal. Because of the complexity and variability of 2D gels, manual intervention is still required for gel image analysis.

Steps in image analysis include the following:

1. Scanning the gel at 300 dpi to acquire a digital TIFF image.
2. Detecting and quantifying all protein spots using 2-D analysis software.
3. Aligning images of different gels in order to group identical spots.
4. Identifying unmatched spots that reflect changes in protein expression or mobility, using 2-D analysis software.
5. Using statistical or clustering analysis to relate changes in protein expression or mobility to functional differences between samples.

Background corrections should be performed before comparing spot intensities. Reliable assignment of a protein spot should require reproducibility in three or more 2-D gels, representing at least two independent samples of the same experimental condition.

It is important to manually verify the spot detection and spot-matching results from the program. Because of variability in gel resolution, staining, and background, automatic image analysis software may not correctly define spot contours, particularly in dense regions of the gel. This variability, together with the complexity in 2-D gel patterns, hinders accurate matching of analogous spots in different gels.

3.8. In-Gel Tryptic Digestion of Proteins in 2-DE Gel Slices

To reduce sample loss during transfer, proteins are digested directly in the polyacrylamide gel matrix *(14,15)*. Trypsin is the enzyme of choice to generate peptides for subsequent analysis by MS (*see* **Note 6**). For best results, wear clean gloves to avoid contamination, use HPLC -grade water and solvents, and carry out all steps in a clean hood (*see* **Note 7**).

1. Using an X-ACTO® knife and blade, excise the protein spot of interest from wet or dry gels and transfer it to a 1.5-mL microcentrifuge tube (nonautoclaved). Alternatively, a pipet tip trimmed at varying lengths from the end with a razor blade can be attached to a P1000 pipettor and used to punch out variable-sized gel spots from a wet gel. It is important to excise only the stained portion of the protein spot and minimize excess gel material. In general, protein recoveries from wet gels are greater than from dried gels.
2. Prepare fresh destaining reagent. Mix equal volumes of 100 m*M* sodium thiosulfate and 30 m*M* potassium ferricyanide, and add 1 mL of the mixture to each gel piece. Cap and agitate for 5 min or until the stain has disappeared. Aspirate the destaining solution.
3. Wash the gel pieces by adding 1.5 mL of HPLC-grade water to each tube. Cap and agitate the tubes at room temperature for 5 min, and then aspirate.
4. Repeat **step 3** two more times.

5. Wash the gel pieces in 500 μL of 100 m*M* ammonium bicarbonate (NH$_4$HCO$_3$) for 20 min with agitation, and then aspirate.
6. Wash the gel pieces in 500 μL of 50% acetonitrile/50 m*M* NH$_4$HCO$_3$ with agitation for 20 min, and then aspirate.
7. In a clean hood, add 50 μL of 100% acetonitrile to shrink each gel piece. Transfer each gel piece with acetonitrile to a clean 0.6-mL microcentrifuge tube. After 10–15 min, remove excess solvent and dry the gel pieces in a Speedvac centrifuge until the gel pieces appear completely dry and opaque (5–10 min).
8. Prepare a stock solution (1 mg/mL) of sequencing-grade, modified trypsin in 50 m*M* acetic acid. Dilute the stock solution 1:50 with 25 m*M* NH$_4$HCO$_3$ and reswell the gel pieces with 10–20 μL of the trypsin solution (0.02 mg/mL). After 15–30 min, remove excess trypsin solution from the tubes and replace with a sufficient volume of 25 m*M* NH$_4$HCO$_3$ to just cover the gel piece (approximately twice the gel volume). The gel pieces must stay wet during the digestion. Incubate overnight at 37ºC.
9. Quench the reaction by adding 1 μL of 88% formic acid to each tube. Sonicate in a bath sonicator for 30 min and transfer the supernatant to new 0.6-mL microcentrifuge tubes. If necessary, speedvac concentrate the supernatant sample to a final volume of 10–20 μL.
10. Remove 0.5 μL of the sample and analyze by MALDI-TOF, as described in **Subheading 3.9.1.** If the sample will not cocrystallize efficiently with matrix, remove one-half of the remaining sample volume and desalt with a ZipTip-C18 column (Millipore) according to instructions, and then reanalyze by MALDI-TOF. If confirming structural information is required, analyze the remaining sample volume by nanoflow ion trap mass spectrometry.

3.9. Peptide Sequencing by MS

MS has become the preferred alternative to Edman degradation and amino acid analysis for characterizing low levels of protein. Mass spectrometers for proteomics applications are equipped with MALDI or electrospray ionization sources.

3.9.1 Peptide Mass Mapping Using MALDI-TOF MS

Peptide samples are mixed with an equivalent volume of a small organic matrix compound (α-cyano-4-hydroxy-*trans*-cinnamic acid) on a stainless steel sample plate and air-dried *(16)*. The mixture forms crystals that are bombarded with photons from a nitrogen laser (337 nm). The benzene rings in the matrix molecules absorb energy from the photons and excite the analyte. Matrix and analyte molecules are desorbed off of the sample plate, creating a plume of gas-phase ions and neutral molecules. It is believed that collisions between the molecules in the explosion plume induce peptide ionization by supporting proton transfer from the matrix. MALDI is most commonly combined with TOF mass spectrometers. Mass-to-charge ratios in TOF mass spectrometers are determined by measuring the time it takes for ions to move through a field-free

flight tube. Assuming a constant accelerating voltage, the flight time for an ion is related to its *m/z* ratio. TOF instruments can detect extremely large ions (in excess of *m/z* 10^6). The sensitivity for peptide analysis is typically in the 25–100 fmo1 range.

Typical operating conditions for the Voyager DE STR TOF-MS are as follows: 20-kV accelerating voltage; 10-V guide wire voltage; 100-ns delay time; 150-scan data averaging; Voyager 5 acquisition software.

When the instrument is calibrated using tryptic peptides resulting from trypsin autolysis as internal standards (842.51 and 2211.10 Daltons), mass accuracy of 50 ppm or better can be observed between 800 and 2500 Daltons. Matrix contaminants dominate spectra below 800 Daltons. The most common nonmatrix contaminants are human keratins from laboratory dust or the investigator. Contaminating peptides from keratins may be observed with singly charged masses (MH+) of 806.39, 992.49, 1002.54, 1089.52, 1105.51, 1117.50, 1164.57, 1200.60, 1233.67, 1356.71, and 1364.63 Daltons. Contaminating peptides from porcine trypsin autolysis may be observed with MH+ of 842.51, 906.50, 952.39, 1006.48, 1045.56, 1469.73, 1736.84, 1768.79, 2158.03, 2211.10, and 2283.18 Daltons.

The monoisotopic peptide masses observed by MALDI-TOF constitute a peptide mass map. Computational tools available on the Internet that use the observed masses to probe databases and identify proteins include ProFound from Rockefeller University (www.expasy.ch/tools/pi_tool.html); MS-Fit from University of California, San Francisco (http://prospector.ucsf.edu); and PeptIdent from Swiss Institute of Bioinformatics (www.expasy.ch/tools/ peptident.html). A commonly used database for search algorithms is the NCBInr (National Center for Biotechnology Information nonredundant) database. Suggested search parameters are as follows: allow one missed cleavage for trypsin, cysteines are modified by iodoacetamide, methionine is partially oxidized (*see* **Note 8**), and a minimum of five peptides is required to match with a mass tolerance for monoisotopic ions under 50 ppm (*see* **Note 9**).

3.9.2. Peptide Sequencing Using Ion Trap MS

Using a syringe, 2–5 μL of the tryptic digest are loaded onto a loop in a microinjection valve (Upchurch). The total amount of sample loaded typically ranges from 25 fmol to several picomoles. Solvent flow from an HPLC is split to provide a final flow rate through the column of 300 nL/min of solvent A (2% acetonitrile in 0.1% aqueous formic acid). The peptides are injected from the loop onto a 10 × 75 μm i.d. fused silica column packed with C18 resin (New Objective) and eluted with a gradient of increasing organic phase reaching 100% solvent B (70/30/0.1 acetonitrile:water:formic acid [v/v/v]) in 15 min. Voltage (1.5–3 kV) is applied to the sample using a microtee at the column

head, inducing spray at the tip of the integral emitter. Gas-phase ions desorb from the sprayed droplets and are directed into the mass spectrometer.

The ion beam passes through two octopoles while a gating lens allows ions to accumulate in the ion trap, a device consisting of two end-cap electrodes and a ring electrode. Voltage applied to the ring electrode is ramped, sequentially ejecting ions with *m/z* values between 300 and 2500 Daltons from the trap in a mass-dependent fashion. Ejected ions are focused into a high-energy collision dynode that emits electrons and secondary particles collected by an electron multiplier. The signal is amplified and a mass/intensity list is sent to an auxiliary CPU and displayed as a mass spectrum. Peptide sequence information is obtained by inducing ions to fragment using additional voltages placed on the end-cap electrodes (MS^2). Higher voltages cause ions to exit the trap, so an ion of interest can be isolated while expelling all others. Lower voltages cause ion trajectories to increase, leading to fragmentation via collisions with helium atoms present in the trap at a pressure of ~1 mTorr. These fragmentation product ions are then ejected by a voltage ramp on the ring electrode. If necessary, a particular fragment ion can be selected for further fragmentation (MS^3).

A typical experiment consists of measuring the mass-to-charge ratio (*m/z*) of ions desorbed from the column eluate (MS), assessing the charge state of the most abundant ions, and then fragmenting them (MS/MS). The instrument automatically performs this experiment. After an ion has been measured two to three times, its *m/z* is placed on an exclusion list so that less abundant ions can be examined (dynamic exclusion), and the experiment is repeated.

Data are analyzed using the Sequest algorithm (ThermoFinnigan). The peptide molecular mass is computed from the *m/z* value of the precursor ion determined in the MS analysis. The peptide molecular mass is then used to search a protein database (Owl, nonredundant, SwissProt, or user determined) parsed into amino acid sequence strings. Theoretical product ions from candidate sequences matching the observed molecular weight are compared with observed product ions. The candidates and observed sequences are then closely compared by cross-correlation. The sequence whose product ion fingerprint most resembles that of the observed data is selected as the top ranked choice. Generally, the match is considered good when a number of the peptides "hit" the same protein, or same family of proteins. When only one peptide (or no peptide) match is observed, the spectra are investigated manually. A sequence tag is constructed and a search through the NCBI database may reveal proteins in other organisms homologous to the protein under investigation.

4. Notes

1. Carbamylation is a common problem associated with the use of urea. Urea in water exists in equilibrium with ammonium cyanate, reacting with amines such

as the N-terminus and ε-amino groups of lysines. Carbamylated proteins exhibit altered p*I*s during focusing and decreased mobility in the SDS-PAGE gel. The reaction also causes artifactual charge heterogeneity, N-terminal blocking, and adduct formation detectable in mass spectra. Avoid carbamylation by using pure-grade urea, never heating the solution above 37ºC (since the cyanate level increases with temperature and pH), and using a cyanate scavenger (primary amine) such as carrier ampholytes.

2. To clean glass plates, soak them in hot laboratory detergent for at least 2 h, and then scrub them with a scouring pad. Rinse off the detergent and store the plates in a dust-free environment until they are used. Before use, wipe each plate with a methanol-soaked tissue.

3. It is very important to prepare 10% APS solution freshly each time gels are polymerized. Old solutions deteriorate and affect the rate of polymerization; hence, the pore size will be different and 2-DE gels will not be reproducible when APS is old.

4. In a standard analytical 2-DE gel loaded with 100–200 µg of protein, the first high-intensity spots should begin to appear within 1 min. Delayed appearance is generally observed when aldehydes are omitted from the fixation step.

5. When the gel is dipped in the developing solution, a brown microprecipitate of silver carbonate should form. This precipitate must be redissolved to prevent it from depositing on the gel and increasing background. Immediately agitating the developing tray redissolves the precipitate.

6. Trypsin cleaves specifically at the C-terminal ends of Lys and Arg amino acids and is particularly useful for generating samples for MS. The spacing of the enzymatic cleavage sites in most proteins typically yields peptides ranging in molecular weight from 500–2500 Daltons, compatible with mass range requirements for most mass spectrometers. In addition, the basic groups at the N-terminal amine and the C-terminal Lys or Arg residue generate doubly charged peptide ions in the electrospray process that facilitate fragmentation at amide bonds to generate singly charged N-terminal (b-type) and C-terminal (y-type) product ions. Tryptic peptides rarely contain internal arginines, minimizing internal fragmentations that complicate product ion mass spectra.

7. Keratin contamination is a significant problem when analyzing samples by MS. Rigorous care should be taken to ensure that keratin is not introduced from skin, hair, and dust. Use gloves and keep the work area and instrumentation clean.

8. Electrophoresis can introduce artifactual protein modifications, such as methionine oxidation or acrylamide adduction to free cysteine sulfhydryls and N-terminal amines. Common artifactual modifications include cysteine-acrylamide (+71 Daltons), oxidized acrylamide (+86 Daltons), β-mercaptoethanol (+76 Daltons), N-terminal acrylamide (+71 Daltons), methionine oxidation (to sulfoxide) (+16 Daltons).

9. Less than 50 ppm mass accuracy is possible to obtain using internal calibration with two to three peptide masses resulting from trypsin autolysis. If only one tryptic peptide is present in the spectrum, internal calibration can be performed by mixing samples with a standard peptide mixture containing bradykinin (904.47

Daltons), angiotensin (1296.69 Daltons), and Glu-fibrinopeptide (1570.68 Daltons).

Acknowledgments

References

1. Shevchenko, A., Jensen, O. N., Podtelejnikov, A. V., et al. (1996) Linking genome and proteome by mass spectrometry: large-scale identification of yeast proteins from two dimensional gels. *Proc. Natl. Acad. Sci. USA* **93,** 14,440–14,445.

2. Soskic, V., Görlach, M., Poznanovic, S., Boehmer, F. D., and Godovac-Zimmerman, J. (1999) Functional proteomics analysis of signal transduction pathways of the platelet-derived growth factor β receptor. *Biochemistry* **38,** 1757–1764.

3. Lewis, T. S., Hunt, J. B., Aveline, L. D., et al. (2000) Identification of novel MAP kinase pathway signaling targets by functional proteomics and mass spectrometry. *Molecular Cell* **6,** 1343–1354.

4. Yu, L. R., Zeng, R., Shao, X. X., Wang, N., Xu, Y. H., and Xia, Q. C. (2000) Identification of differentially expressed proteins between human hepatoma and normal liver cell lines by two-dimensional electrophoresis and liquid chromatography-ion trap mass spectrometry. *Electrophoresis* **21,** 3058–3068.

5. Vercoutter-Edouart, A. S., Lemoine, J., Le Bourhis, X., et al. (2001) Proteomic analysis reveal that 14-3-3 sigma is down-regulated in human breast cancer cells. *Cancer Res.* **61,** 76–80.

6. Stulík, J., Hernychová, L., Porkertová, S., et al. (2001) Proteome study of colorectal carcinogenesis. *Electrophoresis* **22,** 3019–3025.

7. Bjellqvist, B., Ek, K., Righetti, P. G., Gianazza, E., Görg, A., Westermeier, R., and Postel, W. (1982) Isoelectric focusing in immobilized pH gradients: principle, methodology and some applications. *J. Biochem. Biophys. Methods* **6,** 317–339.

8. Görg, A., Postel, W., and Gunther, S. (1988) The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* **9,** 531–546.

9. Wirth, P. J. and Romano, A. (1995) Staining methods in gel electrophoresis, including the use of multiple detection methods. *J. Chromatogr. A* **698,** 123–143.

10. Mann, M., Hojrup, P., and Roepstorff, P. (1993) Use of mass spectrometric molecular weight information to identify proteins in sequence databases. *Biol. Mass Spectrom.* **22,** 338–345.

11. Castellanos-Serra, L., Vallin, A., Proenza, W., Le Caer, J. P., and Rossier, J. (2001) An optimized procedure for detection of proteins on carrier ampholyte isoelectric focusing and immobilized pH gradient gels with imidazole and zinc salts: its application to the identification of isoelectric focusing separated isoforms by in-gel proteolysis and mass spectrometry analysis. *Electrophoresis* **22,** 1677–1685.

12. Switzer, R. C. 3rd, Merril, C. R., and Shifrin, S. (1979) A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. *Anal. Biochem.* **98,** 231–237.

13. Blum, H., Beier, H., and Gross, H. J. (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8,** 93–99.

14. Shevchenko, A., Wilm, M., Vorm, O. and Mann, M. (1996) Mass Spectrometry Sequencing of proteins from silver-stained polyacrylamide gels. *Analyt. Chem.* **68,** 850–858.

15. Gharahdaghi, F., Weinberg, C. R., Meagher, D. A., Imai B. S. and Mische, S. M. (1999) Mass spectrometric identification of proteins from silver-stained polyacrylamide gels: method for the removal of silver ions to enhance sensitivity. *Electrophoresis* **20,** 601–605.

16. Hillenkamp, F., Karas, M., Beavis, R. C., and Chait, B. T. (1991) Matrix-assisted laser desorption/ionization of biopolymers. *Analyt. Chem.* **63,** 1193A–1203A.

19

Practical Methods for Deuterium Exchange/ Mass Spectrometry

Andrew N. Hoofnagle, Katheryn A. Resing, and Natalie G. Ahn

1. Introduction

Deuterium exchange, also called hydrogen exchange, has been used for decades in experiments aimed at elucidating structural information and investigating protein folding *(1–4)*. Deuterium exchange takes place when any acidic proton in a protein molecule exchanges for a deuteron in bulk solvent. Exchange rates for surface-exposed sites depend on factors affecting acidity, while at buried sites, rates depend on the structure and flexibility of the surrounding tertiary structure in permitting access of those protons to bulk solvent. Hydrogen exchange has been detected by ^{3}H incorporation on the whole-protein scale and by nuclear magnetic resonance on the single-proton scale. More recently, deuterium exchange has been interfaced with mass spectrometry (DE-MS) to study large molecules and large multimolecular complexes *(5–7)*. In these experiments, the exchange of a proton for a deuteron is measured as an increase in the mass of a proteolytically generated peptide of one atomic mass unit. More important, when deuterium exchange is analyzed by MS according to the protocol presented here, nearly all side chain hydrogens back-exchange from deuterium to protium during the analysis, so that primarily the backbone amide hydrogens are monitored.

The rate of exchange for backbone amide hydrogens in a properly folded molecule (such as a fully functional kinase at neutral pH) will depend on (1) the primary sequence surrounding the hydrogen, (2) the hydrogen bonding state of the amide hydrogen, and (3) the steric protection of the amide hydrogen from bulk solvent *(8,9)*. The primary sequence is important because local side chains are able to catalyze the deuterium exchange reaction.

Hydrogen bonding and steric protection from solvent reduce the rate of exchange by respectively providing a chemical or a physical barrier to ex-

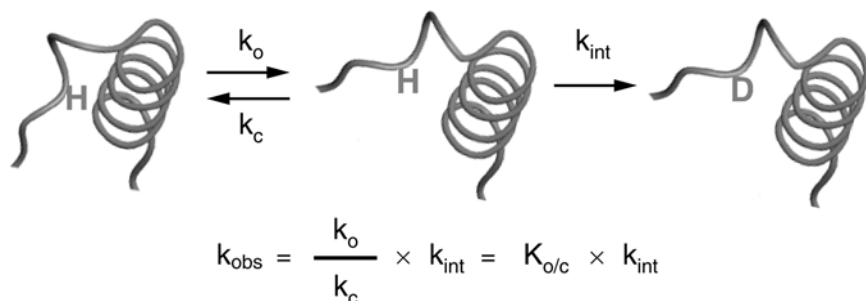$$k_{obs} = \frac{k_o}{k_c} \times k_{int} = K_{o/c} \times k_{int}$$

Fig. 1. Theory of deuterium exchange: a method that reports protein motions.

change. For an amide hydrogen involved in a hydrogen bond and/or buried in the protein to exchange, local structural changes must take place to expose the hydrogen. For native proteins that are folded and fully functional, it is a reasonable first approximation to assume that any local fluctuation in the protein will quickly revert to its initial structure *(4)*. As shown in **Fig. 1**, if we then define an equilibrium between a "closed" exchange–incompetent conformation and an "open" exchange–competent conformation, then the observed rate of deuterium exchange will be equal to the equilibrium constant describing the open and closed states, multiplied by the intrinsic rate of exchange (defined as the rate of exchange of the amide hydrogen in an extended peptide free in solution) *(1,4)*. Therefore, changes in observed deuterium exchange rates may reflect changes in the equilibrium between open and closed conformers or, alternatively, changes in the rates of opening and/or closing.

We have used DE-MS to investigate the changes in the equilibrium of open and closed states in protein kinase molecules in the extracellular signal-regulated kinase 2 (ERK2) mitogen-activated protein kinase (MAPK) pathway following phosphorylation and/or activation *(7,10,11)*. DE-MS studies of ERK2 before and after phosphorylation have revealed evidence for conformational changes in solution that confirm structural changes identified by X-ray crystallography. In addition, DE-MS reveals regions in ERK2 that change in conformational mobility in solution. Because the latter occur within catalytically important regions of the molecule, this suggests that changes in flexibility occur in ERK2 on activation that are important in the catalytic cycle of ERK2.

Besides effects on structure and flexibility, DE-MS allows assessment of intermolecular and localization of protein–protein, protein–nucleic acid, and even protein–small molecule interfaces *(6,12,13)*. In general, one may use qualitative deuterium exchange measurements to monitor changes between any two states, such as unphosphorylated vs phosphorylated, active vs inactive, or bound vs unbound. This chapter describes the application of DE-MS to the
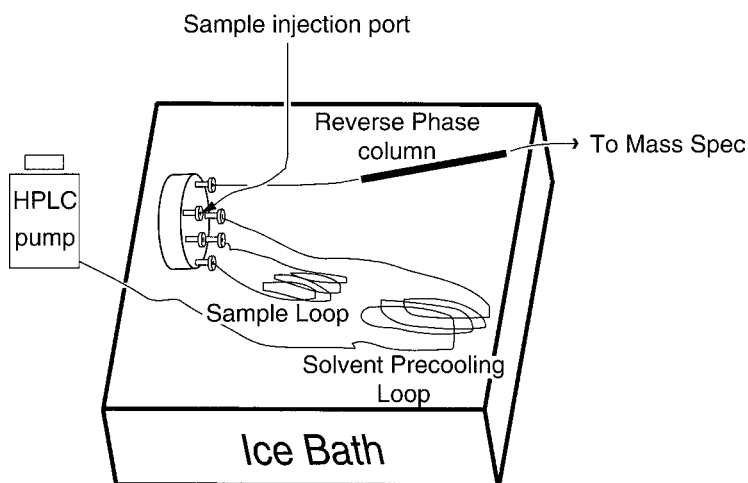
Sample injection port

Fig. 2. Experimental setup for liquid chromatography (LC)/MS of deuterium exchange reaction.

study of large molecules, for the purpose of comparing experimental and control samples, using sample data from previous studies of ERK2.

2. Materials

2.1. Equipment

1. Ice water bath large enough to submerge injector, solvent cooling and injection loops, and high-performance liquid chromatography (HPLC) reverse phase column, as depicted in **Fig. 2**.
2. Salt/dry ice/water bath (–10°C).
3. Hamilton syringe with blunt needle (250 μL); for HPLC injection; the syringe is kept cold on ice and wrapped in a 5-mm-thick layer of laboratory film (Parafilm; American National Can, Chicago, IL) in order to minimize the transmission of heat from fingers to the sample.
4. Polyether ether ketone (PEEK) HPLC injector apparatus (Rheodyne). This injector was chosen for its unique construction materials that minimize the nonspecific association of peptides to the interior surfaces of the apparatus.
5. PEEK injection (1-mL) and solvent cooling (5-mL) loops, respectively used for the pepsin digestion of the deuterated proteins and to cool the solvent before it reaches the column; peptides adhere less to PEEK surfaces than to other plastic surfaces.
6. ABI 140B HPLC pump; any HPLC pump that is able to deliver 20-μL flow rates will work.
7. Capillary (10–15 cm, 500-μm id) HPLC column hand-packed with POROS R120 resin (PerSeptive Biosciences). Columns may be easily and cheaply made from

  fused silica tubing, epoxy, and resin as described in **ref. 14**, or any reverse-phase column of small enough diameter may be used.

8. Standard nebulizer electrospray source.
9. Perkin Elmer Sciex API-III⁺ triple quadrupole mass spectrometer. This model is no longer available for purchase, but any electrospray ionization compatible mass spectrometer will work for data collection in this protocol; similar experiments have been performed using matrix-assisted laser description ionization time-of-flight mass spectrometers and electrospray ion-trap mass spectrometers **(15,16)**.

2.2. Reagents

1. Deuterium oxide (D_2O, Sigma, ≥99.9 atom % D) (Sigma, St. Louis, MO).
2. 25 m*M* Trisodium citrate (Sigma)/25 m*M* disodium succinate (Sigma) buffer, pH 2.40. This is titrated carefully with HCl to precisely pH 2.40, filtered with a 0.22-μm syringe filter, and frozen at –80°C for long-term stability.
3. Pepsin (0.4 mg/mL) in water (Sigma). Pepsin is dissolved in HPLC-grade water and stored frozen in 50-μL aliquots at –80°C; a new aliquot is thawed daily.
4. HPLC buffers:
    a. Buffer A: 0.05% trifluoroacetic acid (TFA) in HPLC grade water (made fresh daily).
    b. Buffer B: 80% HPLC grade acetonitrile, 0.05% TFA in HPLC-grade water (made fresh weekly).
    c. Steps: 7.5, 10, 12.5, 15, 17.5, 20, 22.5, 25, 30, 35, and 40% HPLC-grade acetonitrile, 0.05% TFA in HPLC-grade water (made fresh daily).
5. HPLC-grade acetonitrile.
6. Protein sample (0.5 mg/mL). This should ideally be >90% pure and in an inorganic or organic buffer that lacks amine groups or other Lewis bases that can catalyze the deuterium exchange reaction (*see* **Note 1**).

2.3. Computers and Software

1. MacIntosh computer. MacIntosh computers drive the mass spectrometer and host the MacSpec software (Perkin Elmer Sciex) for data analysis.
2. MacSpec version 3.3 software (Perkin Elmer Sciex), used to analyze the mass spectra.
3. Personal computer with Windows operating system. Any PC that can run Windows 95, 98, 2000, NT, or XP can support the HXPep and the DataFit software (Oakdale Engineering).
4. *Optional:* HXPep software by Dr. Zhongqi Zhang, Amgen, Thousand Oaks, CA, used to estimate back-exchange in peptides in order to correct data.
5. DataFit 6.1 software (Oakdale Engineering; version 7.1 is now available) at www.curvefitting.com/download.htm, used for curve fitting the increase in mass vs time data obtained from the experiment. The program was chosen for its ability to custom tailor different equation models for the system, to test several equations simultaneously, to organize each data set as a project saved to disk, and to easily visualize the data and fitted curves.

3. Methods

3.1. Deuterium Exchange/Mass Spectrometry

The general approach to DE-MS includes incubating the protein sample with D_2O for an in-exchange reaction; quenching the reaction by simultaneously decreasing the pH and the temperature; and digesting the sample with the acid-stable protease, pepsin. The peptides are then separated by reverse-phase HPLC and eluted into the mass spectrometer (*see* **Note 1**). As shown in **Fig. 2**, the HPLC separation of peptides is performed at 0°C in order to reduce the back-exchange, which causes loss of signal owing to exchange of the deuterium incorporated into peptides with hydrogen in the HPLC buffers.

1. Add 90 μL of D_2O (10°C) to 10 μL (5 mg) of protein sample (0°C) (*see* **Note 2**).
2. Incubate at 10°C for varying lengths of time (*see* **Note 3**).
3. Transfer to a –10°C salt/dry ice/water bath for 5 s (*see* **Note 4**).
4. Add 90 μL of 0°C 25 m*M* citrate/25 m*M* succinate pH 2.40.
5. Incubate further in salt/dry ice/water, for a total of 20 s at –10°C.
6. Transfer to ice and immediately add 10 μL of pepsin.
7. Immediately load the protein/pepsin sample (200 μL) at pH 2.40 into the injector loop using a Parafilm-wrapped 250-mL syringe (*see* **Fig. 2** and **Note 5**).
8. One minute after the addition of protease, switch to the inject position in order to inject the digest onto the column.
9. Inject the digest onto the column for 5 min, and then switch the injector back to the load position.
10. Rinse the injector loop with 2 mL of HPLC-grade water (0°C) in order to wash away any D_2O crystals.
11. Load the step gradient by injecting into the loop 40 μL of b uffer B. Then load 17.5 μL of each step buffer into the injection loop in descending order of acetonitrile concentration (e.g., 40, 35, 30, 25, and 7.5%); each buffer is maintained at 0°C prior to loading (*see* **Note 6**).
12. After washing the sample (6 min after **step 9**), reduce the HPLC pump flow rate to 20 μL/min; connect the column to the electrospray source of the mass spectrometer, and switch the injector to inject the gradient onto the column, eluting the peptides into the mass spectrometer (*see* **Note 7**).
13. Before loading another sample, clean the column with 200 μL of HPLC-grade acetonitrile and equilibrate with 4 μL of buffer A at 40 μL/min from the HPLC pump.

3.2. Data Analysis

An example of the analysis of a peptide ion is shown in **Fig. 3**.

1. Open each data file in MacSpec to view the chromatogram.
2. Using the extract ion function, locate an ion of interest.
3. Highlight the extract ion peak and view the mass spectrum containing that ion.
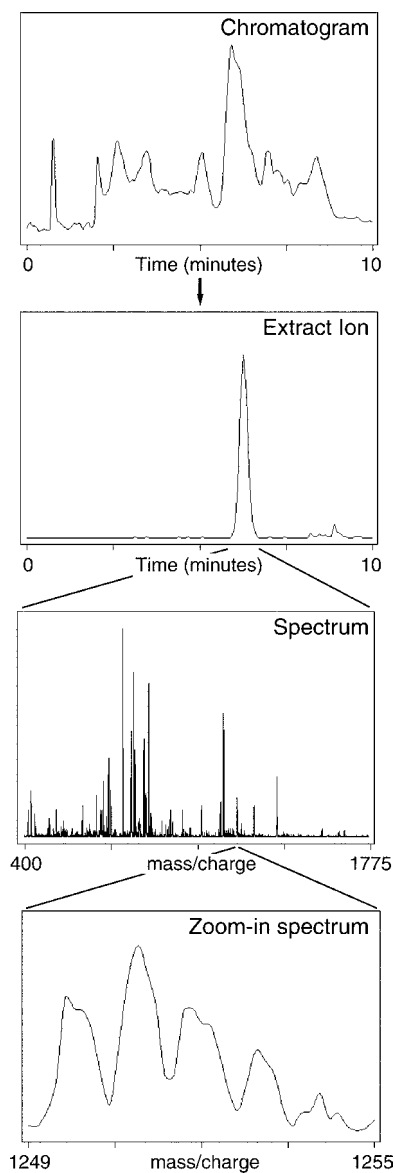4. Zoom in on the ion of interest until it fills the screen.

Fig. 3. Example of the analysis of an LC/MS chromatogram in order to determine average mass/charge of a peptide ion. The chromatogram illustrates the total intensity for each scan vs the scan number (represented by time). The extract ion function extracts and displays both the scans in which the mass/charge of interest is located and the intensity of the mass/charge of interest in those scans. When the scans represented by the extract ion peak are averaged, the spectrum is generated. This average spectrum details the intensity for each ion detected by the mass spectrometer. By zooming in on the peak of interest until it fills the screen, the information to be included in the calculation of the average mass of the peptide can be adjusted.

5. Export the data by pressing command E and selecting a "clipboard"containing the intensities across the peak.

6. Paste the data into a spreadsheet that will calculate the weighted average mass of the ion (*see* **Note 8**)

7. Repeat this for all time points taken.

3.3. Data Correction

3.3.1. In-Exchange

Armed with the time-zero control data (*see* **Note 1**), calculate the artifactual in-exchange-corrected peptide mass for each peptide at each time point using the following equations:

$$M_{t,corr(IE)} = (M_{t,wa} - LM_{\infty,90})/(1 - L) \tag{1}$$

$$L = (M_o - M_{calc})/(M_{\infty,90} - M_{calc}) \tag{2}$$

in which $M_{t,corr(IE)}$ is the artifactual in-exchange-corrected peptide mass at time t, $M_{t,wa}$ is the observed weighted average mass at time t, M_o is the observed mass for the peptide in the time-zero control, M_{calc} is the theoretical average mass of the undeuterated peptide, and $M_{\infty,90}$ is the theoretical mass of the peptide at infinite exchange (with 90% of the backbone amide hydrogens exchanged for deuterium) (*see* **Note 9**).

3.3.2. Back-Exchange

Following digestion and before the peptide masses are analyzed, hydrogens that have in-exchanged for deuterons will slowly exchange back to hydrogen (*see* **Note 10**). We have used three different methods for estimating the fractional back-exchange for each peptide in the sample, and we discuss all three next. Following an estimation of the back-exchange, the data may be corrected by the following equation:

$$M_{t,corr(BE)} = M_{calc} + \left(M_{t,corr(IE)} - M_{calc}\right) / \left(1 - BE\right) \tag{3}$$

in which $M_{t,corr(BE)}$ is the artifactual in-exchange- and back-exchange-corrected mass of the peptide at time t, BE is the fractional back-exchange; $M_{t,corr(IE)}$ is the mass at time t, corrected for in-exchange as in **Subheading 3.3.1.**, and M_{calc} is the theoretical average mass of the peptide.

3.3.2.1. Direct Measurement of Back-Exchange

The back exchange can be directly measured by performing **steps 1–10** in **Subheading 3.1.**, substituting 50 μL of protein sample for 10 μL of protein sample (25 μg protein), and 50 μL of H_2O for 90 μL of D_2O in **step 1**. Rather than loading a step gradient in **step 11**, load the loop with 30 μL of 40% HPLC-

grade acetonitrile, 0.05% TFA in HPLC-grade water, and proceed with **step 12**. However, rather than connecting the column to the mass spectrometer, collect the 30-μL eluent and lyophilize the peptides using a standard lyophilizer. Dissolve the peptides in 30 μL of the buffer used in the deuterium exchange reaction, then add 270 μL of D_2O and heat to 90°C for 90 min. This facilitates the complete deuteration of peptides, which are then cooled to 0°C on ice, and injected into the injector loop (system set up as **Fig. 2**). Inject 100 μL of the deuterated the peptides onto the column after 1 min of incubation in the loop (as in **step 8** in **Subheading 3.1.**), and proceed with **step 9** in **Subheading 3.1.** The fractional back-exchange can then be calculated for each peptide:

$$BE = (M_{\infty,90} - M_{BE})/(M_{\infty,90} - M_{calc}) \tag{4}$$

in which $M_{\infty,90}$ is the theoretical mass of the peptide with 90% of the backbone amide hydrogens exchanged for deuterium (because the deuterium-exchange experiment is performed in 90% D_2O), M_{BE} is the observed mass of the peptide using the back-exchange experiment (in this section), and M_{calc} is the theoretical average mass of the peptide.

3.3.2.2. EMPIRICAL FORMULA

The following equation calculating the fractional back-exchange was derived empirically in our laboratory:

$$BE = \frac{\% \, H_2O}{\% \, D_2O} + \left\{ \frac{\left(\text{peptide elution time from HPLC in min} + 6 \text{ min} \right)}{\times \left[(1\%/\text{min}) / 100 \right]} \right\} \tag{5}$$

in which L is the fraction of artifactual in-exchange at t = o from Eq. 2 and (%H2O/%D2O is the ratio of H_2O to D_2O during proteolysis (*see* **Step 6, Subheading 3.1.**; e.g., 0.55/0.45 for 90% D_2O incubation). The calculation is based on an observed back-exchange of approx 1% for each minute the peptide is on the column prior to elution (*see* **Note 11**).

3.3.2.3. CALCULATION USING THEORETICAL EXCHANGE RATES FROM PEPTIDES FREE IN SOLUTION

The exchange rate for amide backbone hydrogens in peptides free in solution have been determined empirically *(8)*. It is possible to calculate the exchange rate for each peptide in low salt at 0°C with the program HXPep (kindly provided by Dr. Zhongqi Zhang, Amgen). Enter the peptide sequence, choose "NH/D2O" exchange, "oligo" peptide size, "low salt," pH/pD read of

"2.400," "0°C," and "C-terminal considered." This program calculates the rate at which each amide backbone hydrogen will back-exchange. For each backbone amide hydrogen,

$$BE_{amide} = k_{HXPep} \times \left(\text{elution} + \text{wash time}\right) \qquad (6)$$

in which BE_{amide} is the average back-exchange estimate for each backbone amide hydrogen, and k_{HXPep} is the rate of exchange calculated for the amide hydrogen by HXPep. For the whole peptide the fractional back-exchange may be calculated by

$$BE = \Sigma(BE_{amide})/(M_\infty - M_{calc}) \qquad (7)$$

in which $\Sigma(BE_{amide})$ is the sum of BE_{amide} for every amide hydrogen in the peptide, from Eq. 6; M_∞ is the theoretical average mass of the peptide with every backbone amide hydrogen exchanged for deuterium; and M_{calc} is the theoretical average mass of the peptide.

3.4. Curve Fitting

Following the corrections of the data outlined in **Subheadings 3.3.**, the data may be fit using nonlinear least squares curve fitting to a sum of exponentials (*see* **Note 12**). The program we use, DataFit, is extremely versatile and easy to use. It saves to disk all of the work for each peptide, including all of the equations used to fit the data and the statistical analysis of each fit for later reference. An example result of curve fitting is shown in **Fig. 4**.

1. Start a new DataFit project.
2. Input the corrected data ($M_{t,corr(BE)}$ vs time).
3. Under the Solve menu, select "Define User Model…."
4. Select "New…."
5. Enter the name of the equation in Model ID.
6. Enter the equation describing the sum of three exponentials (*see* **Note 12**) under Model Definition:

$$Y = n - a \cdot \exp(-d \cdot x) - b \cdot \exp(-e \cdot x) - c \cdot \text{Exp}(-f - x)$$

7. Similarly, enter the equations describing the sum of two and one exponentials.
8. For each equation, provide initial guesses for the nonlinear least squares algorithm by highlighting the Model ID in the User Defined Models window and clicking on "Rules…" (*see* **Note 13**).
9. Close the User Defined Models window and select Regression under the Solve menu.
10. In the next window, select "Groups" and "Non-linear" and click on "OK."
11. Select "User Defined Groups" in the next window and click "OK" (*see* **Note 14**).

$$Y = N - Ae^{-k_1 t} - Be^{-k_2 t}$$

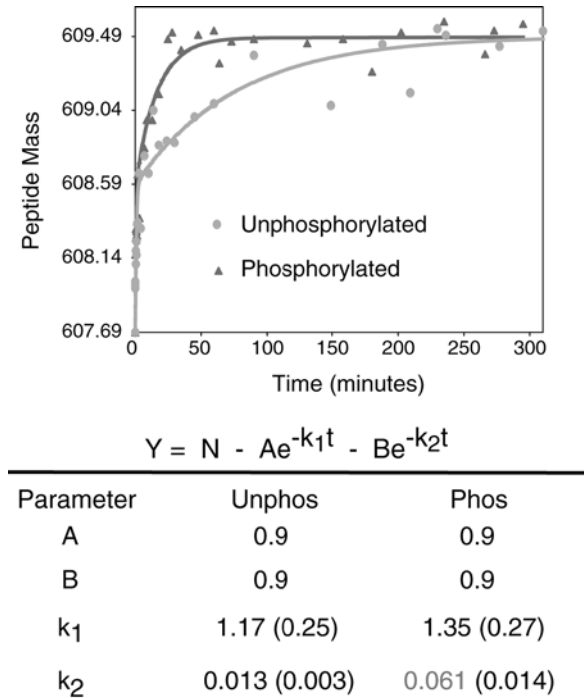| Parameter	Unphos	Phos
A	0.9	0.9
B	0.9	0.9
k_1	1.17 (0.25)	1.35 (0.27)
k_2	0.013 (0.003)	0.061 (0.014)

Fig. 4. Example results obtained from curve fitting. The corrected data from a peptide in ERK2 (unphosphorylated vs phosphorylated) are shown with their fitted curves. When the parameters for the first two pre-exponential weighting factors are "fixed" at 0.9, the rates of deuterium exchange can be directly compared. In this example, the increase in observed exchange may be modeled to an increase in one amide hydrogen exchange rate of approximately fourfold.

4. Notes

1. Several aspects of the experiment should be optimized before the deuterium exchange reaction is performed. First, the buffer conditions of the exchange reaction should be optimized to minimize protein denaturation, while keeping the salt and buffer concentrations as low as possible in order to protect the mass spectrometer and to minimize buffer/salt-catalyzed hydrogen exchange. We have found that 5 mM sodium phosphate (pH 7.0), 50 mM NaCl in the exchange reaction produces low back-exchange. Second, the length of the pepsin digestion should be optimized to generate the greatest number of peptides in the size range of 8–15 amino acids, which yields optimal resolution for exchange measurements. Peptides should be short enough to enable mass resolution, but long enough to bind to the HPLC column and provide some sequence overlap. Third, the step gradient, while resembling that presented in the methods, should be optimized to enable good separation of peptides and elute all peptides from the column in 10 min.

Before performing experiments in D_2O, trial digestions in water should be carried out, following the same experimental protocol outlined in **Subheading 3.1.** Begin the protocol by adding 90 μL of water instead of D_2O in **step 1**, and proceed through **step 11**. Pepsin concentrations, digestion times, and the acetonitrile concentrations used in the step gradient should be varied to produce optimal peptide lengths and separation. Following this optimization, trial runs are carried out using tandem LC/MS/MS in order to determine the sequence of each peptide. Because pepsin is a nonspecific protease, peptides must be sequenced *de novo* by matching peptide mass and sequence tag information to the primary protein sequence.

Following quenching and digestion and before washing the peptides on the column (**steps 4–9**), deuterium will in-exchange with peptide backbone protons to a low extent. To correct for the amount of this "artifactual in-exchange," perform three control measurements ("time-zero controls") by adding 90 μL of quench buffer to 10 μL of protein sample at 0°C and adding the acidified protein sample to 90 μL of D_2O that was just transferred to a –10°C water bath. After incubating the acidified sample with D_2O at –10°C for 20 s, proceed with **steps 6–12**.

2. Reactions are carried out at 10°C to reduce the rate of in-exchange to ranges measurable in the time frame of the experiment, which has a dead time of 7 s. For faster rate measurements, others have used rapid freeze-quench apparatuses to perform exchange reactions in the millisecond range *(17)*.

3. We have successfully sampled exchange times ranging from 7 s to 5 h. The shortest time points are limited by the manual speed required to manipulate the sample, and the longest time points are limited by the stability of the protein. A method for determining from the deuterium exchange experiment if the protein remains properly folded during the experiment is to characterize the mass spectral peaks. If a subpopulation of the protein is unfolded, some peptide mass/charge peaks will become "double peaks," where the emergence of a higher mass peak, whose average mass corresponds to peptide that is 90% in-exchanged at the backbone amide hydrogens, indicates stable, complete solvent exposure of an ordinarily protected peptide. Alternatively, the protein can be tested for specific activity following incubation in D_2O.

4. Dry ice is added in small increments, while monitoring a thermometer in the bath to maintain constant temperature. The sample is incubated at –10°C briefly enough to rapidly cool to 0°C, but not long enough to freeze the solution. In the past, we have used a –15°C bath in order to cool down the samples, which minimizes back-exchange to 12%. However, the solution would frequently crystallize when injected into the loading loop. To avoid this problem, we now use a –10°C water bath.

5. Before this point, ensure that the apparatus is set up as shown in **Fig. 2**. The ice-water slurry should have a large ice pack (~3 cm) over the water/ice layer. The column is immersed in the water/ice layer; typically, we see higher back-exchange as the proportion of water to ice increases. The HPLC pump should be delivering a steady flow of 40 μL/min of buffer A. *See* **step 13** for proper preparation of the column.

6. A step gradient is used because it reduces the amount of time needed to deliver the gradient to the column by reducing the dead volume between the pump. It also makes it easier to sequence many peptides at once in a single LC/MS run (*see* **Note 1**). The acetonitrile concentrations at each step are chosen empirically, based on pre-experimental trials to optimize peptide separation.

7. The mass spectrometer is operated according to standard procedures with the following parameters: the plenum chamber is near room temperature (24 to 25°C), the detector is calibrated with polypropylene glycol before the measurements, the orifice voltage is set to 75 V and the curtain gas to 0.9 (L/min), and the data are collected by scanning a 0.15 Da window from 400 to 1775 Da with a 0.55-ms dwell time (5.1 s/scan).

8. When the data are exported from MacSpec, they are exported as binomial data, the first and second columns enumerating the mass/charge (in Da) and the signal intensity of that mass/charge detected by the mass spectrometer, respectively. The weighted average mass/charge detected for each ion of each peptide can then be evaluated using any spreadsheet program utilizing the following equation:

$$M_{t,wa} = \{[\Sigma(m/z \times \text{intensity})/\Sigma(\text{intensity})] \times z\} - z \tag{7}$$

in which $\Sigma(m/z \times \text{intensity})$ is the mass/charge of each ion (column 1) multiplied by the signal intensity of each ion (column 2) summed over all ions detected; and $\Sigma(\text{intensity})$ is the sum of the intensities of all ions, or the total intensity of the peptide (the sum of the values in column 2).

9. For a derivation of Eqs. 1 and 2, *see* **ref. 10**.

10. A standard technique for measuring back-exchange involves incubating the protein sample in D_2O for 24 h at 30°C to achieve complete in-exchange, then digesting the sample with pepsin and monitoring the loss of mass during measurement, analogous to technique 1. However, because large proteins, such as protein kinases, contain regions that do not exchange after 24 h, and because they frequently denature on incubation at 30°C for long periods, we have tested alternative techniques for the estimation of the back-exchange in DE-MS experiments. In **Subheadings 3.3.2.1.–3.3.2.3.**, we present three techniques that we have tested for estimating back-exchange in large molecules not amenable to prolonged incubation at higher temperatures with D_2O. We have found that all three techniques yield the same back-exchange estimates (5% variability) for >95% of the peptides in ERK2. The most serious drawback of technique 1 is that not all peptides are recovered; thus, a fraction of the peptides will need to be estimated using technique 2 or 3. Because of its simplicity, we recommend technique 3, which agrees with previous theory and is independent of the laboratory performing the experiment.

11. For the derivation of Eq. 5, *see* **ref. 10**.

12. As described in **Subheading 1.**, DE-MS can be used in a variety of experiments. However, its most general application is to assess the qualitative differences between two similar samples, e.g., to assess changes in flexibility or the localization of bimolecular or multimolecular interactions. Therefore, it is important to

determine whether two curves are indeed statistically different from one another. Nonlinear least squares curve fitting provides a flexible method for determining whether curves are distinct and can occasionally be exploited one step further in describing the magnitude of observed rate changes, which to a first approximation reflects the thermodynamic differences between two states of a protein molecule. The time courses are modeled by a sum of exponentials, in which each amide hydrogen theoretically exchanges with a deuteron at a given rate. In theory, each amide backbone hydrogen would be represented by a separate exponential term:

$$Y = N_{th} - e^{-k_1 t} - e^{-k_2 t} - e^{-k_3 t} - e^{-k_4 t} \ldots - e^{-k_w t} \tag{8}$$

in which Y is the mass of the peptide, w is the total number of amide hydrogens minus the amino terminal amide hydrogen, k_1, k_2, k_3, k_4, and k_w are the rates of exchange for the different amide hydrogens at each residue (units: time-unit^{-1}), t is time, and N_{th} is the theoretical mass of the peptide when 100% of the amide hydrogens are in-exchanged (if the exchange reaction were carried out in 100% D_2O). However, in practice, amide hydrogen exchange rates are averaged into fast, intermediate, and slow rates, and time courses can be fit to between one and three exponential terms. In addition, not every amide exchanges over the time course (e.g., 0–5 h), leaving a number of nonexchanging amides (*NE*). The resulting model is written as follows:

$$Y = N - Ae^{-k_1 t} - Be^{-k_2 t} - Ce^{-k_3 t} \tag{9}$$

in which N is the mass of the peptide at the end of the time course and is equal to $M_{calc} + [0.9 \times (w - NE)]$, in which Mcalc is the theoretical average mass of the peptide and w is the total number of amide hydrogens in the peptide minus the number of proline amides; and A, B, and C correspond to the number of amide hydrogens (multiplied by 9) exchanging at the averaged exchange rates k_1, k_2, and k_3, respectively.

13. Nonlinear least squares curve-fitting algorithms need initial guesses in order to begin. The choice of the initial guesses should not determine the outcome of the fit, which is generally true if there are enough data points. Therefore, several different starting guesses should be tested for convergence to the same fit. N can be estimated by using the average mass of the last few data points in the time course. Because the exchange reaction is performed in 90% D_2O, and because the data have been corrected for the back-exchange and the artifactual in-exchange, the number of amides that are exchanging in the peptide should be $P = [0.9 \times (w - NE)]$. Therefore, the preexponential weighting factors (a, b, and c in the DataFit equation; *see* **Subheading 3.4., step 6**) should sum to P and any combination of weighting factors where $a + b + c = P$ should suffice. The observed rates of hydrogen exchange in this protocol typically range from >10 min^{-1} to <0.001 min^{-1}, and guesses within this range will usually converge to a least-squares fit of the data. In general, the success of a model depends on the initial guesses of N and a, b, and c the most. To save the equations for use with

other peptides, Export the User Defined Model equations in the User Defined Models window of the DataFit program.

14. There are several ways to use curve fitting in order to assess the statistical relevance of various models of hydrogen exchange. For instance, some peptides will overlap with one another in primary protein sequence. The data obtained from these peptides partially comprise exchange rates for identical backbone amide hydrogens. Therefore, parameters from fits of some peptides may be used as "fixed" parameters in overlapping peptides. To do this, select "Regression" from the Solve menu. In the Solution Setup window, select "Single Model." In the Single Model Regression Setup window, check the "User Specified" box and then click on the "Specify…" button. Enter the initial value to which the parameter will be fixed (e.g., a value from another fit), and select "Constant" for the IC Type.

However, when applying parameters fit to one peptide to the fit of an overlapping peptide in this way, it is important to verify that the overlapping peptides are generated at constant levels throughout the in-exchange time course. Because a hydrogen bond containing a deuterium atom will be weaker than a hydrogen bond containing a proton, it is possible that the secondary structure of the protein will vary in some places as the in-exchange reaction proceeds. In such a case, the cleavage pattern of pepsin may change with time. One way to monitor the cleavage pattern is to analyze the signal intensity of the peptides recovered vs time. If the cleavage pattern changes, some peptides will decrease in total signal intensity and others will increase, precluding comparison of overlapping peptides.

Another way to exploit nonlinear least squares is to test the null hypothesis that the curves are identical. If the curves are identical (within error) to one another, it will be possible to fit the two sets of data with the same equation. For instance, if a peptide measured in two different experimental states has two amide hydrogens exchanging over the time course, then fixing the a and c values in both samples to 0.9 in nonlinear least squares curve fitting will generate rates of exchange for each of those amides in each peptide that will be directly comparable to one another. It will also provide a measure of the error of the fitted rates, which can be used to determine whether the curves and thus the rates are indeed different.

In some peptides, the peptide mass will have reached an apparent plateau at late times. In other cases, the mass of the peptide may still increase slowly at the end of the time course, attributed to the presence of a very slowly exchanging amide in the peptide. The latter can be modeled by nonlinear least squares fitting of the curve by determining the slope of the last few data points, and fixing that slope to the rate of exchange (e.g., as d, e, or f in the above User Defined Model in DataFit) of one amide hydrogen.

Finally, it is possible that fitted weighting factors (a, b, and c) will not sum to N. This is the result of one of two problems: (1) the data are of poor quality and the fitted curve fails to pass through both the zero time point (M_{calc}) and the asymptotic last data points, or (2) the back-exchange estimate from **Subheading 3.3.2.** is slightly incorrect. In the first example, holding N or the preexponential weighting factors (a, b, and c) constant during the fit will solve the problem. In the

second case, one may adjust the estimated *BE* in order for the difference in the mean of the last few data points and the time-zero control to be a multiple of 0.9.

References

1. Englander, S. W. and Kallenbach, N. R. (1984) Hydrogen exchange and structural dynamics of proteins and nucleic acids. *Quart. Rev. Biophys.* **16,** 521–655.
2. Smith, D. L. and Zhang, Z. (1994) Probing noncovalent structural features of proteins by mass spectrometry. *Mass Spectrom. Rev.* **13,** 411–429.
3. Clarke, J. and Itzhaki, L. S. (1998) Hydrogen exchange and protein folding. *Curr. Opin. Struct. Biol.* **8,** 112–118.
4. Li, R. and Woodward, C. (1999) The hydrogen exchange core and protein folding. *Protein Sci.* **8,** 1571–1590.
5. Hoofnagle, A. N., Resing, K. A., and Ahn, N. G. (2003) Protein analysis by hydrogen exchange mass spectometry. *Annu. Rev. Biophys. Biomol. Struct.* **32,** 1–25.
6. Wang, F., Blanchard, J. S., and Tang, X. (1997) Hydrogen exchange/electrospray ionization mass spectrometry studies of substrate and inhibitor binding and conformational changes of *Escherichia coli* dihydrodipicolinate reductase. *Biochemistry* **36,** 3755–3759.
7. Resing, K. A. and Ahn, N. A. (1998) Deuterium exchange mass spectrometry as a probe of protein kinase activation: analysis of wild-type and constitutively activate mutants of MAP kinase kinase-1. *Biochemistry* **37,** 463–475.
8. Bai, Y., Milne, J. S., Mayne, L., and Englander, S. W. (1993) Primary structure effects on peptide group hydrogen exchange. *Proteins* **17,** 75–86.
9. Bai, Y, Englander, J. J., Mayne, L., Milne, J. S., and Englander, S. W. (1995) Thermodynamic parameters from hydrogen exchange measurements. *Methods Enzymol.* **259,** 344–356.
10. Resing, K. A., Hoofnagle, A. N., and Ahn, N. G. (1999) Modeling deuterium exchange behavior of ERK2 using pepsin mapping to probe secondary structure. *J. Am. Soc. Mass Spectrom.* **10,** 685–702.
11. Hoofnagle, A. N., Resing, K. A., Goldsmith, E. J., and Ahn, N. G. (2001) Changes in protein conformational mobility upon activation of extracellular regulated protein kinase-2 as detected by hydrogen exchange. *Proc. Natl. Acad. Sci. USA* **98,** 956–961.
12. Mandell, J. G., Falick, A. M., and Komives, E. A. (1998) Identification of protein-protein interfaces by decreased amide proton solvent accessibility. *Proc. Natl. Acad. Sci. USA* **95,** 14,705–14,710.
13. Andersen, M. D., Shaffer, J., Jennings, P. A., and Adams, J. A. (2001) Structural characterization of protein kinase A as a function of nucleotide binding: hydrogen-deuterium exchange studies using matrix-assisted laser desorption ionization-time of flight mass spectrometry detection. *J. Biol. Chem.* **276,** 14,204–14,211.
14. Resing, K. A. and Ahn, N. G. (1997) Protein phosphorylation analysis by electrospray ionization-mass spectrometry. *Methods Enzymol.* **283,** 29–44.

15. Mandell, J. G., Falick, A. M., and Komives, E. A. (1998) Measurement of amide hydrogen exchange by MALDI-TOF mass spectrometry. *Anal. Chem.* **70,** 3987–3995.
16. Deng, Y., Pan, H., and Smith, D. L. (1999) Selective isotope labeling demonstrates that hydrogen exchange at individual peptide amide linkages can be determined by collision-induced dissociation mass spectrometry. *J. Am. Chem. Soc.* **121,** 1966,1967.
17. Dharmasiri, K. and Smith, D. L. (1996) Mass spectrometric determination of isotopic exchange rates of amide hydrogens located on the surfaces of proteins. *Anal. Chem.* **68,** 2340–2344.

20

Use of *Xenopus* Oocytes and Early Embryos to Study MAPK Signaling

Eusebio Perdiguero and Angel R. Nebreda

1. Introduction

   Xenopus oocyte maturation is a powerful system to study biochemical mechanisms that regulate intracellular signaling and cell-cycle control. Fully grown *Xenopus* oocytes are arrested at the G2/M boundary of the first meiotic division. The process of maturation can be induced in vitro by incubation of isolated oocytes with hormones, such as the natural inducer progesterone or others such as insulin or insulin growth factor-1. Several hours after hormone treatment, the nucleus (germinal vesicle) migrates to the animal pole of the oocyte, and the nuclear envelope dissolves. This process is known as germinal vesicle breakdown (GVBD) and can be easily scored because it produces the appearance of a white spot on the animal pole of the oocyte by displacement of the pigments. The maturing oocyte progresses through meiosis I and then enters meiosis II, where it remains arrested at the metaphase II, awaiting fertilization. The early signaling pathways activated by progesterone are not well understood (for reviews, *see* **refs. *1*** and ***2***). However, a key enzymatic activity that regulates the G2/M transition is the maturation promoting factor (MPF), which is composed of cyclin B and the serine/threonine protein kinase Cdc2. This complex is maintained in an inactive form (pre-MPF) during the G2 arrest owing to the phosphorylation of Cdc2 by the Myt1 protein kinase. The dephosphorylation necessary to activate pre-MPF and induce progression of the oocyte into meiosis is catalyzed by the phosphatase Cdc25C. Several pathways have been described to regulate the phosphorylation-dephosphorylation of Cdc2, and hence, they also regulate MPF activity *(2)*. The mitogen-activated protein kinase (MAPK) pathway composed of the protein kinase Mos, the MAPK kinase MEK1, the extracellular signal-regulated kinase (ERK)-like MAPK XMpk1, and the protein kinase Rsk is likely to be involved in the inhibition of Myt1 and

thus responsible (at least in part) for MPF activation. Treatments that interfere with the activation of the MAPK cascade either delay or block Cdc2 activation and therefore oocyte maturation. Conversely, activation of the MAPK pathway can cause Cdc2 activation and oocyte maturation in the absence of progesterone.

The role of the ERK MAPKs in meiotic maturation has been extensively studied. By contrast, very little is known about the possible role of the other MAPK subfamilies c-Jun N-terminal kinase (JNK) and p38 in *Xenopus* oocyte maturation and early embryonic development. Recently, Bagowski et al. *(3)* have shown that the JNK pathway is activated during oocyte maturation and may be involved in the process. The role of the p38 subfamily in *Xenopus* oocyte maturation remains unknown. Note, however, that the *Schizosaccharomyces pombe* p38 MAPK Sty1/Spc1 can influence mitotic initiation *(4,5)*, that the sea star p38 homolog Mipk is inactivated during G2/M progression *(6)*, and that the human p38α and p38β have been implicated in the G2/M checkpoint after ultraviolet radiation *(7)*.

2. Materials

2.1. Frogs

The South-African clawed frog *Xenopus laevis* can be purchased from several companies worldwide. We have successfully used frogs purchased from *Xenopus* I (Dexter, MI; www.Xenopusone.com), Nasco (Fort Atkinson, WI; www.nascofa.com), CNRS breeding center (Rennes, France; www.cnrs.fr/ SDV/xenopes.html), and African Reptile Park (South Africa). For reviews on the materials and conditions needed to maintain and develop a healthy frog colony, *see* **refs. *8–10***.

2.2. Equipment

1. Narishige PN-30 micropipet (needle) puller and watchmaker's forceps.
2. Injection needles: Narishige GD-1 borosilicate glass capillaries (1 × 90 mm)
3. Narishige IM 300 Motor Drive microinjector. Compressed gas (water-free nitrogen) is used as the source of pressure.
4. CELL-VU hemocytometer cover slip to measure volume of injected material.
5. Aluminum foil, ice bucket, sharp razor blade or scissors, and blunt-end forceps.
6. Protein electrophoresis and semidry blotting apparatuses (Hoefer).

2.3. Reagents and Kits

1. MEGAscript in vitro transcription kit; can be obtained from Ambion.
2. Cap analog [m7G(5')ppp(5')G] (New England Biolabs) stock prepared at 40 m*M* in water and stored at –20°C.
3. Phenol-chloroform extraction reagents and isopropanol; these should be prepared for the mRNA purification as in **Subheading 3.1.**

4. Glutathione Sepharose beads and glutathione (Pharmacia), for purification of the recombinant protein (*see* **Subheading 3.2.**).
5. Pregnant mare serum gonadotropin (PMSG). 3-aminobenzoic ethyl ester acid (Sigma), and Collagenase B from *Clostridium hystoliticum* (Roche Diagnostics Gmbh) are used to obtain oocytes (*see* **Subheading 3.3.**).
6. Human chorionic gonadotropin and gentamycin are used to obtain eggs (*see* **Subheading 3.4.**).
7. Progesterone (Sigma: stock solution of 5 mg/mL, prepared in ethanol and stored at –20°C) is used to induce oocyte maturation (*see* **Subheading 3.6.**).
8. Gel electrophoresis nitrocellulose membranes (Protran, 0.22 µm; Schleicher/Schuell), nonfat dry milk, primary antibodies, secondary antibody (horseradish peroxidase [HRP]-coupled; Dako, or infrared-fluorescent-labelled, Molecular probes and Li-Cor) and enhanced chemiluminescence (ECL) detection system (Amersham), for immunoblotting (*see* **Subheading 3.9.**).
9. Protein A–Sepharose beads (Pharmacia), for immunoprecipitation (*see* **Subheading 3.10.**).
10. Antibodies to phosphorylated and total ERK and other components of the MAPK cascades should be used to determine the activity of the various components as in **Subheadings 3.14.–3.16.**

2.4. Buffers

1. Modified Barth saline medium (mBarth): 15 mM HEPES, pH 7.6, 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO$_3$, 0.3 mM Ca(NO$_3$)$_2$, 0.4 mM CaCl$_2$, 0.8 mM MgSO$_4$, streptomycin, and penicillin-G at 10 µg/mL each. This medium is prepared as two separate stock solutions. Solution A includes 128 g of NaCl, 2 g of KCl, 5 g of NaHCO$_3$, and 89 g of HEPES/L of water, with the final pH adjusted to 7.6 using NaOH. Solution B includes 1.9 g of Ca(NO$_3$)$_2$, 2.25 g of CaCl$_2$, and 5 g of MgSO$_4$ in 1 L of water. The stocks are stored at room temperature. The antibiotics are prepared together as a stock solution of 10 mg/mL and stored at –20˚C. To prepare 1X mBarth, mix 40 mL each of solutions A and B with 1 mL of antibiotic stock and take to 1 L using distilled water.
2. MMR: 5 mM HEPES, pH 7.8, 100 mM NaCl, 2 mM KCl, 1 mM MgSO$_4$, and 2 mM CaCl$_2$. Store at 4°C. It can be prepared as a 10X stock solution *(11)*.
3. Microinjection buffer: 20 mM Tris-HCl pH 7.5, 50 mM NaCl.
4. Histone H1 kinase (H1K) buffer: 80 mM β-glycerophosphate, pH 7.5; 20 mM EGTA, 15 mM MgCl$_2$, 2.5 mM benzamidine, and 2 µg/mL each of aprotinin and leupeptin. Store at –20°C. Before use add fresh 1 mM dithiothreitol (DTT) and 1 mM 4-2-aminoethyl-benzene-sulfonyl fluoride (AEBSF) or phenylmethyl-sulfonyl fluoride (PMSF).
5. Immunoprecipitation (IP) buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5–1% Nonidet P-40, 5 mM EGTA, 5 mM EDTA, and 20 mM NaF. Store at 4°C. Before use add 0.1 mM Na vanadate, 1 mM PMSF, 2 µM microcystin, 2.5 mM benzamidine, and 10 µg each of aprotinin, leupeptin, and pepstatin A/mL.
6. TTBS buffer: 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween-20.

Prepare as a 10X stock solution. Store at room temperature.

7. Dialysis buffer: 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, and 5% glycerol. Prepare before use.

7. RNA sample buffer: 3 mL of 10X MOPS/EDTA (0.2 M MOPS, pH 7.0, 10 mM EDTA, and 50 mM Na acetate in diethylpyrocarbonate [DEPC]-treated water), 15 mL of formamide, 5 mL of 37% formaldehyde, 2 mL of 37% glycerol, 1.6 mL of 2% bromophenol blue, and 2 mL of DEPC-treated water. Store at –20°C. Add 10 μL/mL ethidium bromide to the working solution. All reagents should be RNase free.

8. Stock solutions: 1 M DTT; 0.1 M AEBSF; 1 mM NaVanadate; 1 mM microcystin; 0.1 M PMSF; 0.5 M benzamidine; and 10 mg/mL of aprotinin, leupeptin, and pepstatin A. Store at –20°C.

3. Methods

3.1. Preparation of mRNAs

The cDNAs are cloned into plasmid or phagemid vectors that contain promoters recognized by the highly specific bacteriophage SP6, T3, or T7 DNA-dependent RNA polymerases (*see* **Note 1**). Plasmid DNA (typically 1 to 2 μg or 10 μL of a miniprep) is linearized with a restriction enzyme that cuts downstream of the insert, avoiding the use of restriction enzymes that leave 3' overhanging ends (we used *Xba*I or *Xmn*I for FTX5). In vitro-transcribed mRNAs can be obtained using the MEGAscript in vitro transcription kit or similar in vitro transcription systems. The transcription reaction is performed according to the manufacturer's instructions, except that the concentration of guanosine triphosphate is reduced to 1.5 mM and each reaction is supplemented with 3 mM Cap analog [m7G(5')ppp(5')G]. The mRNA is purified by phenol-chloroform extraction and then precipitated with isopropanol. The quality and amount of the mRNA can be assessed by standard 1% TAE agarose gel electrophoresis, using RNA sample buffer and heating the sample 5 minutes at 65°C before loading. As a reference, RNA markers (0.28–6.58 kb; Promega, Madison, WI) can be used.

3.2. Preparation of Recombinant Proteins

The proteins for injection into oocytes are expressed in *E. coli* and purified following standard procedures. Usually the recombinant proteins have a tag, such as encoding maltose-binding protein (MalE), glutathione S-transferase (GST), or 6 histidines, which facilitate purification by affinity chromatography. A typical purification procedure for a GST-tagged protein would be as follows:

1. Grow the *E. coli* strain BL21 (DE3) containing the appropriate expression plasmid in LB medium plus 100 μg/mL of ampicillin (or the appropriate antibiotic) to an optical density of 0.8, and then induce expression of the protein by adding 0.1 mM IPTG.

2. After 4–16 h at room temperature, harvest the bacteria from 500-mL culture, and

resuspend in 13.5 mL of cold phosphate-buffered saline (PBS) containing protease inhibitors (PMSF, aprotinin, and leupeptin) plus 1 mg/mL of lysozyme, and sonicate.

3. Add ice-cold Triton X-100 (1.5 mL from a 10% stock) to give a final concentration of 1%, mix, and centrifuge at 5000*g* for 20 min at 4°C.
4. Incubate the supernatant with 1 mL of glutathione Sepharose beads for 1 h at 4°C.
5. Wash the beads three times with cold PBS and once with 50 m*M* Tris, pH 8.0, and elute the protein with freshly prepared 10 m*M* glutathione in 50 m*M* Tris, pH 8.0.
6. Dialyze purified proteins overnight against 2–5 L of dialysis buffer and store in aliquots at –70°C.

3.3. Xenopus *Oocyte Preparation*

1. Prime frogs 3–7 d before use with 50 U of PMSG.
2. Anesthetize a frog by immersing in ice-cold water or in a solution of 0.4% 3-aminobenzoic ethyl ester acid (Sigma) for 30–40 min.
3. Once the animal is not reacting to any external stimulus, lay it on its back on a clean, cold surface (e.g., glass or aluminum foil over an ice bucket). With a sharp razor blade or scissors, make a small incision above the leg on one side of the frog's abdomen (posterior and ventral side) to expose the ovary tissue.
4. Using blunt-end forceps, gently pull out the ovary, cut it into small pieces with the scissors, and put it in a glass Petri dish containing mBarth.
5. Wash the tissue several times with mBarth to eliminate rests of blood. For more detailed information about oocyte isolation and frog surgery, *see* **refs. 8** and **9**.
6. When a large number of oocytes are needed (*see* **Note 2**), it is usually more convenient to do enzymatic defolliculation with collagenase. Cut the ovary tissue into small pieces with sharp scissors and then incubate in mBarth medium containing 0.5–1 mg/mL of collagenase for about 1 h at room temperature. The incubation time required varies among individual frogs and different batches of collagenase; an excess of collagenase digestion can make the oocytes very fragile and therefore more difficult to handle. It is important to note that calcium-free medium can be used to attenuate the activity of collagenase, instead of mBarth.
7. After collagenase treatment, thoroughly wash the oocytes with large volumes of 0.1 *M* NaCl and finally with mBarth. Maintain the oocytes in a glass Petri dish with mBarth at 18°C, changing the medium frequently to avoid cloudiness. Collagenase treatment releases oocytes of all the stages of oogenesis. Sorting of the right oocytes for the following experiments is described in **Note 3**.

3.4. Xenopus *Embryo Preparation*

1. To induce female frogs to lay eggs, inject 500 U of human chorionic gonadotropin (Sigma) into the dorsal lymph sac of primed frogs (**Subheading 3.3.**). Then store the frogs in a medium with 100 m*M* NaCl and 5 μg/mL of gentamycin for 16 h. Under these conditions the frogs should naturally lay eggs.
2. After overnight incubation, monitor the frogs in order to quickly collect and transfer the layed eggs into a clean glass Petri dish. For detailed procedures of egg collection techniques, *see* **ref. 9**.
3. Eggs are fertilized in vitro using male testes. Sacrifice the male frog by immers-

of the two pigmented areas, and for embryos, inject preferentially in the region that corresponds to the future dorsal part (ventral to the gray crescent). *See* **Note 6** for more information.

7. When mRNA is injected, keep the oocytes/embryos in 1X mBarth overnight at 16–18°C to increase the translation efficiency. *See* **Note 7** for more information.

3.6. *Analysis of Microinjected Oocytes and Embryos*

The microinjected oocytes and embryos can be analyzed by different methods depending on the goal of the experiment and the proteins being studied. The most common techniques are described below.

3.6.1. *Western Blot*

1. Transfer a number of oocytes (normally 10) to a 1.5-mL Eppendorf tube, remove the excess of liquid with a drawn-out Pasteur pipet, and add 10 μL/oocyte of cold lysis buffer (*see* **Note 8**).

2. Homogenize the oocytes by pipetting up and down, and maintain on ice to avoid protein degradation. The yolk proteins are the most abundant in *Xenopus* oocytes and embryos. These proteins (43 and 31 kDa), together with the actin (42 kDa), can create problems if the protein under study has a similar molecular weight.

3. To eliminate most of the yolk proteins, centrifuge the samples at 13,000*g* for 10 min at 4°C.

4. After the centrifugation, three phases can be distinguished: a lipid phase (top), a cytosolic phase (middle), and the yolk platelets and cell debris (bottom). Transfer the cytosolic phase to a new Eppendorf tube (*see* **Note 9**).

5. Mix the samples with the standard protein sample buffer and boil for 5 min before loading onto a sodium dodecyl sulfate-polyacrylamide gel. Use 0.5–1 oocyte equivalent per lane.

6. The proteins are now ready to be separated by electrophoresis and analyzed by Western blot with the appropriate antibodies, following standard protocols.

3.6.2. *Labeling of Oocytes*

To study protein synthesis or phosphorylation, oocytes can be labeled by incubation with $[^{35}S]$ methionine or with $[^{32}P]$ orthophosphoric acid, respectively.

1. Inject 50 nL of $[^{35}S]$ methionine (1 mCi/mL) per oocyte, and incubate for 2–4 h at room temperature.

between the animal and the vegetal poles and then transfer to fresh mBarth for recovery.

7. After 30–60 min, remove the oocytes damaged as a consequence of the injection procedure.

8. To score for cleavage arrest, inject the embryos at the two- or four-cell stage (Stages 2 and 3), generally in one of the two or four blastomeres.

3.6. Scoring for Oocyte Maturation: GVBD

1. For the induction of meiotic maturation, sort stage VI oocytes and incubate in 5 µg/mL (15.9 µ*M*) of progesterone. We normally use six-well Petri dishes containing 4 mL of mBarth/well.

2. Add the oocytes to the well (routinely 20–60 oocytes) taking care not to increase the volume, and then add 4 µL of the progesterone stock. An equivalent group of oocytes is used as control (without progesterone).

3. After overnight incubation at 18°C, score the maturation by the appearance of a white spot at the animal pole of the oocyte.

4. Confirm GVBD cytologically by investigating the presence of the nucleus after fixation in 3% TCA or biochemically by assaying MPF activation, either by the dephosphorylation of Cdc2 or by the in vitro phosphorylation of the MPF substrate histone H1 (*see* **Subheading 3.12.**).

5. For biochemical characterization, transfer the oocytes to Eppendorf tubes, remove the medium, and either lyse or immediately freeze the oocytes in liquid nitrogen, and then stored at –80°C.

6. For TCA treatment, fix oocytes in ice-cold 3% TCA for at least 5–10 min. To detect the presence of the nucleus in the oocyte, use thin forceps to make a section in the animal pole, at an angle of about 45°C from the equator, and remove the resulting small animal pole cap. This should show a round, white nucleus in G2-arrested oocytes, whereas in mature oocytes (after GVBD), only the yellowish cytoplasm is seen. This method can also be used to differentiate a normal maturation with GVBD from the so-called pseudo-maturation, where the oocytes present a more or less defined white spot, sometimes mislocalized, but still having a nucleus inside.

7. As an alternative, boil oocytes for 2 min in PBS and then process exactly as after the treatment with TCA.

3.7. Preparation of Oocyte and Embryo Lysates

1. Prepare lysates by crushing fresh or frozen oocytes and embryos with a plastic or wooden stick in 10 µL of H1K buffer per oocyte/embryo (or 20 µL of IP buffer for immunoprecipitations).

2. Centrifuge the extract for 10–15 min at 4°C in a microcentrifuge (14,000*g*).

3. Transfer the cytosolic supernatant to fresh ice precooled Eppendorf tubes and use directly or freeze at –70°C.

3.8. Scoring for Embryo Cleavage Arrest

Score the arrest of the injected embryo blastomeres by following the cell divisions, which occur every 30 min. It is also possible to record embryo development using a time-lapse video system. To differentiate between mitotic and interphase arrest, a kinase assay with the lysate toward histone H1 can be done. Mitosis-arrested cells have increased cyclin-dependent kinase activity and therefore an increased ability to phosphorylate histone H1.

3.9. Immunoblotting

1. Separate oocyte and embryo lysates by sodium dodecyl sulfate (SDS)-PAGE on a 15% Anderson gel *(13)*, using the equivalent of one oocyte or embryo per lane.
2. After electrophoresis, transfer proteins to nitrocellulose membranes (Protran 0.22 μm; Schleicher & Schuell) using a semidry blotting apparatus (Hoefer).
3. Block the membrane for 1 h at room temperature in TTBS buffer supplemented with 5% nonfat dry milk, and then incubate for 2–6 h at room temperature or overnight at 4°C with the corresponding antibody in TTBS supplemented with 1% milk or 3% bovine serum albumin.
4. After washing three times in TTBS for 10 min each, incubate the membrane with the secondary antibody (HRP-coupled; Dako) in TTBS supplemented with 1% milk for 1 h at room temperature. Perform detection using an ECL detection system (Amersham).
5. Alternatively, detection can be performed with the Odyssey Infrared Imaging System (Li-Cor), which requires the use of secondary antibodies labeled with Alexa Fluor 680 (Molecular Probes) or Li-Cor IRDye 800 (Rockland).

3.10. Immunoprecipitation

1. Prepare lysates in IP buffer as described in **Subheading 3.7.** and further dilute the supernatant in IP buffer to 0.3–0.5 ml. Perform all the incubations at 4°C.
2. To do a first precleaning step to reduce the background, incubate with 25 μL of protein A–Sepharose beads for 30 min followed by a spin at 200*g* for 2 min.
3. Take the supernatant to another Eppendorf tube and mix with the antibody (typically 2 to 3 μL of rabbit serum or 1–4 μg of purified antibody), and then rock for at least 2 h at 4°C.
4. Add 20 μL of protein A- (or protein G-) Sepharose beads and rock for 1 h at 4°C.
5. Recover the beads by spinning at 200*g* for 2 min. The supernatant can be used for another IP or to confirm the depletion of the protein.
6. Wash the beads two to three times with 1 mL of IP buffer and one to two times with 1 mL of H1K buffer, and finally resuspend in the appropriate buffer for kinase assay or SDS-PAGE analysis.

3.11. Pull-Down Experiments

1. For pull-downs, dilute 1:1 in IP buffer 200 μL of oocyte lysate prepared as described in **Subheading 3.7.**, and mix with 2–5 μg of recombinant protein

prebound to 15 μL of the corresponding beads (amylose, glutathione). As a control, beads alone or beads bound to the corresponding tag (malE, GST) should be used.

2. After 1 h of rocking at 4°C, wash the beads three times with IP buffer, and one time with H1K buffer.

3. Resuspend the beads in H1K buffer and analyze by immunoblot or kinase assay. For more details, *see* **Note 6**.

3.12. Kinase Assays

The kinase assay can be done using total oocyte/embryo lysates (4 μL; the equivalent of half an oocyte/embryo) or samples from immunoprecipitation or pull-down experiments. Typically, 1–3 μg of the protein substrate is incubated for 15 min at room temperature in a final volume of 12 μL of H1K buffer supplemented with 2 μCi of $[^{32}P\text{-}\gamma]$ATP (3000 Ci/mmol) and 50 μM cold ATP. The kinase reactions are analyzed by SDS-PAGE and autoradiography and, if required, quantified by PhosphorImager analysis. Kinase reactions can also be performed with only cold ATP and analyzed by immunoblotting using antibodies that specifically recognize the phosphorylated substrate.

3.13. Biochemical Analysis of Oocyte Maturation

As mentioned earlier, oocyte maturation can be biochemically scored by assaying the activation of MPF. The kinase activity of MPF can be measured using histone H1 as an in vitro substrate. The kinase assay is performed as described in **Subheading 3.12.**, using 3 μg of histone H1 (Sigma, Histone type III-S from calf thymus; stock prepared at 10 mg/mL in water and stored at –20°C). Histone H1 can be visualized in SDS-PAGE gels as a doublet of ~31 kDa (**Fig. 1**). A complementary way to score for MPF activation is to follow the electrophoretic mobility shift that accompanies activation of Cdc2, the MPF kinase subunit. The nature of the shift is explained in **Note 7**. Aliquots of oocyte lysates (equivalent to one oocyte) are immunoblotted as described in **Subheading 3.9.** We normally used the anti-Cdc2 3E1 mouse monoclonal antibody (MAb) generated by J. Gannon and T. Hunt (Cancer Research UK Clare Hall Laboratories, South Mimms, UK). In G2-arrested oocytes, two Cdc2 bands can be detected by immunoblotting, whereas in mature oocytes, the upper band (corresponding to cyclin B-bound and Tyr-phosphorylated Cdc2) is downshifted concomitant with the increase in H1 kinase activity of the lysates (**Fig. 1**). For more details, *see* **Note 8**.

3.14. MAPKs in Oocyte Maturation

The activity of the ERK MAPK p42 XMpk1 can be measured by in vitro kinase assay using 3 μg of MBP (Sigma, Myelin basic protein from bovine brain; stock prepared at 10 mg/mL in water and stored at –20°C) as a substrate,
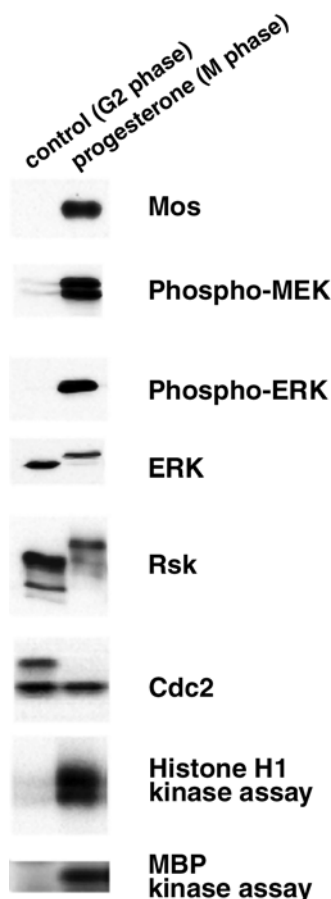
Fig. 1. Activation of MPF and ERK MAPK during *Xenopus* oocyte maturation. Lysates were prepared from oocytes untreated (control) or treated with progesterone overnight (progesterone) and analyzed by immunoblotting using anti-Mos[Xe], antiphospho-MEK1/2, antiphospho-p44/42 ERK MAPK, anti-Rsk1 + anti-Rsk2, or anti-Cdc2 antibodies, as described. The kinase activity of the lysates was also assayed using histone H1 and MBP as in vitro substrates for MPF and MAPK, respectively.

as described in **Subheading 3.12.** MBP can be visualized in SDS-PAGE gels as a band of about 20 kDa (**Fig. 1**; **Note 9**).

The activation of ERK MAPKs can also be detected by immunoblot using antiphospho antibodies that specifically recognize p44 and p42 ERK MAPKs phosphorylated on Thr202 and Tyr204 (e.g., the E10 mouse monoclonal from Cell Signalling Technology) (*see* **Fig. 1**). Alternatively, it is possible to visualize the phosphorylated and active ERK MAPK owing to the reduced electro-

phoretic mobility in SDS-PAGE gels. We use an antibody generated against the 13 carboxyl-terminal amino acids of *Xenopus* XMpk1 MAPK *(14)* (**Fig. 1**). Other commercially available antibodies against mammalian ERK2 are likely to recognize also the *Xenopus* protein.

The activation of other components of the ERK MAPK cascade in *Xenopus* oocytes can be detected by immunoblotting (**Fig. 1**). Synthesis of the MAPK kinase kinase Mos can be detected using the rabbit polyclonal antibody C237 (Santa Cruz Biotechnology). To detect activation of the MAPK kinase MEK1, we use an antibody that recognizes the Ser217 and Ser221 phosphorylated and active form of MEK1/2 (Cell Signalling Technology). Rsk activation can be followed by the shift in the electrophoretic mobility that occurs on phosphorylation and activation, using for example the polyclonal antibodies anti-Rsk1 (C-21) and anti-Rsk2 (C-19; Santa Cruz Biotechnology). Alternatively, kinase assays can be performed after immunoprecipitation. The Rsk protein can be immunoprecipitated using anti-Rsk1/Rsk2 antibodies prebound to protein-G beads and assayed using recombinant GST-Myt1 protein as a substrate *(14)*. Mos and MEK immunoprecipitations followed by kinase assay are also possible using the appropriate antibodies and substrates *(15–18)*.

3.15. Study of p38 MAPKs in Xenopus *Oocytes*

In addition to the study of endogenous ERK MAPKs, *Xenopus* oocytes are useful to study regulation and function of ectopically expressed proteins. We describe here the methodology that we are using to study ectopically expressed p38 MAPKs. Similar approaches could be used for other protein kinases.

cDNAs encoding p38α and p38γ MAPK isoforms were cloned in the FTX5 expression vector. A constitutively active form of the p38 MAP kinase activator MKK6, named MKK6-DD was cloned in the vector FTX4, which is exactly the same as FTX5 but without the Myc tag *(19)*. Following the procedures described above, mRNAs are generated from these constructs and injected into stage VI oocytes that are incubated overnight to allow expression of the proteins. Oocyte lysates are then prepared and analyzed by immunoblotting and kinase assays. The expression levels of the p38 MAPKs are tested using anti-Myc 9E10 mouse MAb, which is commercially available from many suppliers. The levels of MKK6 expression are tested using an anti-MKK6 rabbit polyclonal antibody *(19)*. The activation of the expressed p38 MAPKs by MKK6 can be detected by immunoblot with phosphospecific antibodies, such as the dual phospho-p38 antibody from Cell Signalling Technology or Sigma (**Fig. 2**). The kinase activity of the proteins is tested by in vitro kinase assay as described in **Subheading 3.12.** using the carboxyl-terminal tail of the transcription factor ATF2 (amino acids 19–96) fused to GST *(20)* as a substrate (**Fig. 2**). This can be performed in total oocyte lysates. However, because of
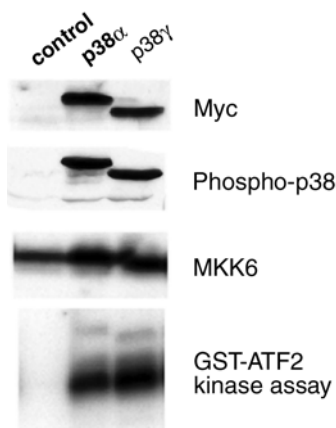
Fig. 2. Expression and activation of p38 MAPKs in *Xenopus* oocytes. The mRNAs for MKK6-DD and p38α or p38γ (Myc tagged) were coinjected into oocytes, which were incubated overnight to allow expression of the proteins. Uninjected oocytes were also processed in parallel (control). Lysates were prepared and analysed by immunoblotting using anti-Myc, antiphospho-p38 MAPK, or anti-MKK6 antibodies, as described. The kinase activity of the lysates was also assayed using GST-ATF2 as in vitro substrate for p38 MAPKs.

the presence of other kinases that can phosphorylate ATF2 (especially in mature oocytes), it is more accurate to assay p38 MAPK activity in immunoprecipitates prepared using anti-Myc antibody coupled to beads (commercially available, e.g., from Santa Cruz Biotechnology). For more details, *see* **Note 10**.

3.16. Induction of Embryo Cleavage Arrest by MAPKs

The role of the ERK MAPK pathway inducing cleavage arrest in *Xenopus* embryos has been well documented. Injection of Mos, in vitro–activated recombinant ERK2 MAPK, or constitutively active Rsk mutants can all efficiently arrest cell division in embryos *(21–23)*. The ERK MAPK pathway is also likely to play a physiologic role in CSF arrest of mature oocytes at metaphase II of the meiotic cell cycle *(24,25)*. The ectopic expression of p38α MAPK has also been reported to induce mitotic arrest in *Xenopus*-cleaving embryos *(26)*, although with significantly less efficiency than ERK MAPKs.

As described in **Subheadings 3.5.** and **3.8.**, MKK6-DD and p38 MAPK mRNAs or the corresponding recombinant proteins are injected in two- or four-cell embryos, and the development is monitored for 4 to 5 h to detect cleavage arrest. The expression and activation of the injected p38 MAPKs can be scored as described for the oocytes, either by immunoblot with anti-Myc and

antiphospho-p38 antibodies or with a kinase assay using GST-ATF2 as substrate (*see* **Note 11**).

4. Notes

1. We normally use the FTX5 plasmid *(27)* that contains an amino-terminal Myc tag (to allow detection and immunoprecipitation with the anti-Myc MAb 9E10), but there are other possibilities such as pCS2+ *(28)* or pSP64T *(29)* and derivatives.

2. There are two different systems to obtain individual oocytes from the ovarian tissue (defolliculation): manual and enzymatic. Manual defolliculation may be more difficult and requires some training, although it can be useful for the isolation of a small number of oocytes (for a description of manual defolliculation, *see* **refs. *9*** and ***30***).

3. In our experiments we use only the fully grown, stage VI oocytes, so it is necessary to sort them from the mixture. Stage VI oocytes can be distinguished from stage V oocytes; they have slightly larger diameter (1.2–1.3 mm) and normally present a white interphase (like a ring) in the equator between the animal and vegetal poles. It is very important that the oocytes selected for injection have a homogeneous color and perfect shape, without any scars.

4. The time of dejellying (membrane removal) depends on the batch of eggs and should be monitored carefully to avoid damaging the embryos.

5. It is important to also inject some oocytes with the sample buffer as a control. Solutions containing detergents or more than 150 mM salt should be avoided. Typical amounts for injection may be 10–50 ng of protein and 1–20 ng of mRNA. In the case of mRNAs, there is usually a good correlation between the concentration of mRNA injected and the amount of protein expressed in the oocyte. Before injection, mRNA is normally tested for expression in a rabbit reticulocyte translation system.

6. Pull-down experiments are used to isolate interacting proteins, such as to identify the substrate or activators of a purified MAPK, or, conversely, to identify the MAPK that binds a possible substrate or activator *(19,31)*. Note, however, that only some interactions with MAPKs are strong enough to allow detection by pull-down experiments.

7. This shift is owing to the dephosphorylation of Cdc2 on Tyr15 (and probably also Thr14) and always correlates with faster migration of the Cdc2 protein in SDS-PAGE gels.

8. To obtain good resolution of the two Cdc2 bands, we use 20-cm-long 15% acrylamide Anderson gels. Antibodies that crossreact with *Xenopus* Cdc2 can be purchased from several commercial suppliers (e.g., the A17 MAb from Santa Cruz Biotechnology or a rabbit polyclonal antibody from Cell Signalling Technology). It is also possible to follow the dephosphorylation of Cdc2, by using Phospho-Cdc2 Tyr15 specific antibodies (Cell Signalling Technology).

9. For visualization of MBP it is better to use 20% Anderson or 15% Laemmli gels. To increase specificity, the assay can be performed after immunoprecipitation with MAPK-specific antibodies as described in **Subheading 3.10.**

10. The ability to express and activate p38 MAPKs allows investigation of the interplay between p38 MAPKs and other signaling pathways involved in oocyte maturation. Moreover, *Xenopus* oocytes can also be used to study mechanistic aspects of p38 MAPK signaling, such as by coexpression of different p38 MAPK isoforms together with specific activators and targets, at different levels and incorporating specific mutations.

11. It is more complicated and time-consuming to work with embryos than with oocytes. Nevertheless, the assay using cleaving embryos provides an alternative and, to some extent, complementary system to the meiotic maturation of oocytes.

Acknowledgments

References

1. Ferrell, J. E., Jr. (1999) Xenopus oocyte maturation: new lessons from a good egg. *Bioessays* **21,** 833–842.
2. Nebreda, A. R. and Ferby, I. (2000) Regulation of the meiotic cell cycle in oocytes. *Curr. Opin. Cell Biol.* **12,** 666–675.
3. Bagowski, C. P., Xiong, W., and Ferrell, J. E., Jr. (2001) c-Jun N-terminal kinase activation in Xenopus laevis eggs and embryos: a possible non-genomic role for the JNK signaling pathway. *J. Biol. Chem.* **276,** 1459–1465.
4. Millar, J. B., Buck, V., and Wilkinson, M. G. (1995) Pyp1 and Pyp2 PTPases dephosphorylate an osmosensing MAP kinase controlling cell size at division in fission yeast. *Genes Dev.* **9,** 2117–2130.
5. Shiozaki, K. and Russell, P. (1995) Cell-cycle control linked to extracellular environment by MAP kinase pathway in fission yeast. *Nature* **378,** 739–743.
6. Morrison, D. L., Yee, A., Paddon, H. B., Vilimek, D., Aebersold, R., and Pelech, S. L. (2000) Regulation of the meiosis-inhibited protein kinase, a p38(MAPK) isoform, during meiosis and following fertilization of seastar oocytes. *J. Biol. Chem.* **275,** 34,236–34,244.
7. Bulavin, D. V., Higashimoto, Y., Popoff, I. J., et al. (2001) Initiation of a G2/M checkpoint after ultraviolet radiation requires p38 kinase. *Nature* **411,** 102–107.
8. Lacal, J. C. (1998) Oocytes microinjection assay to study the MAP-kinase cascade. *Methods Mol. Biol.* **84,** 139–152.
9. Sive, H. L., Grainger, R. M., and Harland, R. M. (2000) *Early Development of Xenopus laevis: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
10. Wu, M. and Gerhart, J. (1991) Raising Xenopus in the laboratory. *Methods Cell Biol.* **36,** 3–18.
11. Murray, A. W. (1991) Cell cycle extracts. *Methods Cell Biol.* **36,** 581–605.
12. Keller, R. (1991) Early embryonic development of Xenopus laevis. *Methods Cell Biol.* **36,** 61–113.

13. Anderson, C. W., Baum, P. R., and Gesteland, R. F. (1973) Processing of adenovirus 2-induced proteins. *J. Virol.* **12,** 241–252.

14. Palmer, A., Gavin, A. C., and Nebreda, A. R. (1998) A link between MAP kinase and p34(cdc2)/cyclin B during oocyte maturation: p90(rsk) phosphorylates and inactivates the p34(cdc2) inhibitory kinase Myt1. *EMBO J.* **17,** 5037–5047.

15. Sagata, N., Oskarsson, M., Copeland, T., Brumbaugh, J., and Vande Woude, G. F. (1988) Function of c-mos proto-oncogene product in meiotic maturation in Xenopus oocytes. *Nature* **335,** 519–525.

16. Kosako, H., Gotoh, Y., Matsuda, S., Ishikawa, M., and Nishida, E. (1992). *Xenopus* MAP kinase activator is a serine/threonine/tyrosine kinase activated by threonine phosphorylation. *EMBO J.* **11,** 2903–2908.

17. Nebreda, A. R., Hill, C., Gomez, N., Cohen, P., and Hunt, T. (1993) The protein kinase mos activates MAP kinase kinase in vitro and stimulates the MAP kinase pathway in mammalian somatic cells in vivo. *FEBS Lett.* **333,** 183–187.

18. Nebreda, A. R. and Hunt, T. (1993) The c-mos proto-oncogene protein kinase turns on and maintains the activity of MAP kinase, but not MPF, in cell-free extracts of Xenopus oocytes and eggs. *EMBO J* **12,** 1979–1986.

19. Alonso, G., Ambrosino, C., Jones, M., and Nebreda, A. R. (2000) Differential activation of p38 mitogen-activated protein kinase isoforms depending on signal strength. *J. Biol. Chem.* **275,** 40,641–40,648.

20. Livingstone, C., Patel, G., and Jones, N. (1995) ATF-2 contains a phosphorylation-dependent transcriptional activation domain. *EMBO J.* **14,** 1785–1797.

21. Haccard, O., Sarcevic, B., Lewellyn, A., et al. (1993) Induction of metaphase arrest in cleaving Xenopus embryos by MAP kinase. *Science* **262,** 1262–1265.

22. Kosako, H., Gotoh, Y., and Nishida, E. (1994) Mitogen-activated protein kinase kinase is required for the mos-induced metaphase arrest. *J. Biol. Chem.* **269,** 28,354–28,358.

23. Gross, S. D., Schwab, M. S., Lewellyn, A. L., and Maller, J. L. (1999) Induction of metaphase arrest in cleaving Xenopus embryos by the protein kinase p90Rsk. *Science* **286,** 1365–1367.

24. Sagata, N. (1996) Meiotic metaphase arrest in animal oocytes: its mechanisms and biological significance. *Trends Biol. Cell* **6,** 22–28.

25. Tunquist, B. J. and Maller, J. L. (2003) Under arrest: cytostatic factor (CSF)-mediated metaphase arrest in vertebrate eggs. *Genes Dev.* **17**, 683–710.

26. Takenaka, K., Moriguchi, T., and Nishida, E. (1998) Activation of the protein kinase p38 in the spindle assembly checkpoint and mitotic arrest. *Science* **280,** 599–602.

27. Howell, M. and Hill, C. S. (1997) XSmad2 directly activates the activin-inducible, dorsal mesoderm gene XFKH1 in Xenopus embryos. *EMBO J.* **16,** 7411–7421.

28. Rupp, R. A., Snider, L., and Weintraub, H. (1994) Xenopus embryos regulate the nuclear localization of XMyoD. *Genes Dev.* **8,** 1311–1323.

29. Krieg, P. A., and Melton, D. A. (1984) Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs. *Nucleic Acids Res.* **12,** 7057–7070.

30. Smith, L. D., Xu, W. L., and Varnold, R. L. (1991) Oogenesis and oocyte isolation. *Methods Cell Biol.* **36,** 45–60.

31. Gavin, A. C. and Nebreda, A. R. (1999) A MAP kinase docking site is required for phosphorylation and activation of p90(rsk)/MAPKAP kinase-1. *Curr. Biol.* **9,** 281–284.

21

MAPK Cascades in the Brain

Lessons From Learning

Diego E. Berman and Yadin Dudai

1. Introduction

The mitogen-activated protein kinase (MAPK) signaling cascades (including the extracellular signal-regulated kinases [ERKs], the c-Jun N-terminal kinases [JNKs], and the p38 stress-activated protein kinases) are abundant in neurons in the mature central nervous system *(1)*. Originally, these cascades were discovered as critical regulators of cell division and differentiation in nonneuronal cells, raising the question: What role would these cascades play in nondividing, terminally differentiated neurons in the adult brain?

Recent data have led to the view that MAPKs can function as biochemical signal integrators and coincidence detectors in response to extracellular signals in neurons, subserving processes such as synaptic plasticity and learning in the adult brain *(2–4)*. Activation of ERKs has been implicated in the induction of long-term potentiation (LTP), an extensively studied form of synaptic plasticity, in area CA1 in the hippocampus *(5)*, and in several learning paradigms in the rat, such as conditioned taste aversion *(2,6)*, fear conditioning *(7)*, and spatial learning *(8)*. Moreover, the activation of ERKs, but not JNKs, has been shown to modulate the activation of the transcription factor Elk-1 in the rat insular cortex on exposure of rats to a novel taste *(6)*.

A critical aspect in the aforementioned studies is measurement of activation of the MAPK cascades after electrophysiologic stimulations or behavioral manipulations. MAPK activation is most conveniently determined by a technique that combines brain tissue dissection and Western blots *(3,6,7)* with specific antibodies that recognize the biphosphorylated activated forms of several members of the MAPK cascades (these specific phosphosite antibodies are commercially available from several companies).
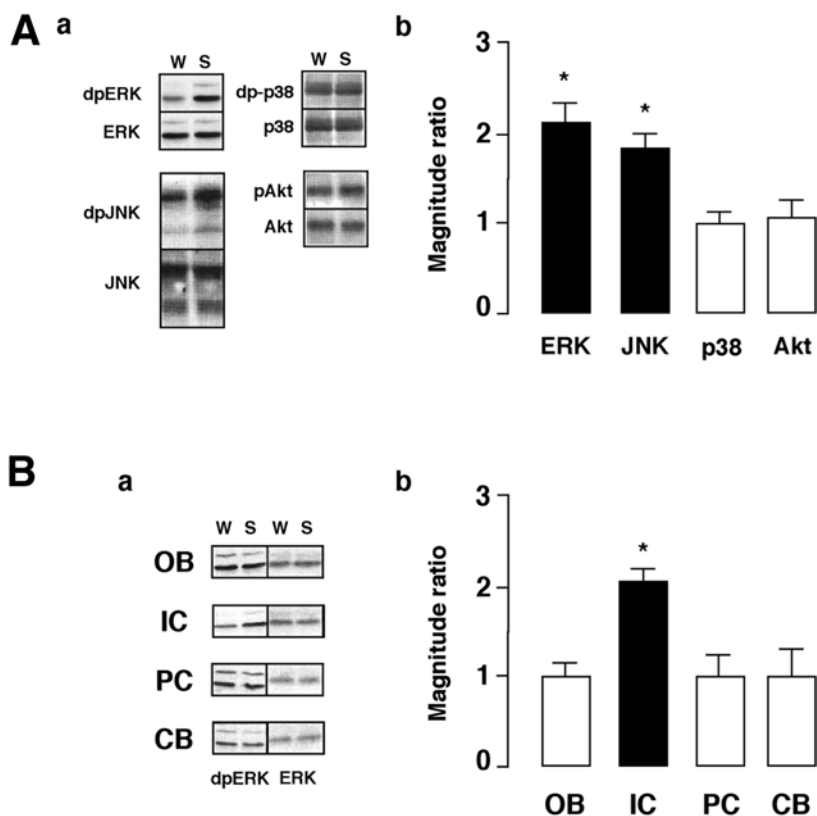
Fig. 1. Detection of activation of several MAPK cascades in brain. **(A,a)** Representative blots showing effect of gustatory experience (as described under **Subheading 3.**) on the ERK (p42 and p44), JNK, p38, and Akt signaling cascades in the insular cortex (IC). The IC was dissected out 30 min after the offset of the drinking period. **(A,b)** Quantification of results obtained in (a). The magnitude ratio is expressed as the phosphorylated-forms MAPK (saccharin)/phosphorylated-forms MAPK (water). **(B, a)** Level of activated ERK (dpERK) and total ERK (ERK) in various brain regions 30 min after exposure to taste. **(B,b)** Quantification of results obtained in (a). OB, olfactory bulb; PC, piriform cortex; CB, cerebellum. S, animals exposed to saccharin; W, animals exposed to water; $n = 8$. $*p < 0.05$.

An example of the data derived using this combination of animal behavioral manipulations and the sensitive Western blotting procedure is shown in **Fig. 1**. Activation of ERKs and JNKs, but not p38 or the Akt kinase, is induced by exposure of rats to 10 mL of a solution of an unfamiliar taste (e.g., 0.1% saccharin) during an incidental learning situation in the context of conditioned

taste aversion. The effect is observed specifically in the insular cortex, which contains the taste cortex.

2. Materials

2.1. Behavioral Manipulations

1. Male Wistar rats (~60 d old, 250–300 g) are used. They are caged individually at 22 ± 2°C in a 12-h light/dark cycle.
2. 0.1% (w/v) Sodium saccharin solution.
3. Glass pipets (10 mL).

2.2. Tissue Dissection and Homogenization

1. Guillotine.
2. Glass-Teflon homogenizer (small, approx 0.5 mL).
3. Sodium dodecyl sulfate (SDS) sample buffer: 10% glycerol, 5% β-mercaptoethanol, and 2.3% SDS, in 62.5 m*M* Tris-HCl, pH 6.8.

2.3. Protein Determination and Electrophoresis

1. Lowry reagents *(9)*:
   a. Reagent A: 2% Na_2CO_3 in 0.1 *N* NaOH.
   b. Reagent B_1: 2% sodium potassium tartar.
   c. Reagent B_2: 1% $CuSO_4$.
   d. Reagent C: 50 mL of reagent A + 0.5 mL of reagent B_1 + 0.5 mL of reagent B_2; prepare fresh.
   e. Reagent D: Folin reagent (commercially available).
2. Bovine serum albumin (BSA) solution, 0.25 mg/mL.
3. 0.4 *N* NaOH solution.
4. Electrophoretic chamber and power supply unit.
5. SDS-polyacrylamide gel electrophoresis running buffer (for a 10X solution: 30 g of Tris, 141 g of glycine, 10 g of SDS, to a final volume of 1000 mL).
6. Polyacrylamide gels (7.5–8%) in Tris-HCl buffer (pH 6.8 for upper Tris buffer, pH 8.8 for lower Tris buffer).
7. Protein standards (in a 10- to 200-KDa range, e.g., Rainbow markers from Amersham).

2.4. Western Blotting and Autoradiography

1. Transfer buffer: 30 g of Tris, 18.75 g of glycine, up to a final volume of 5 L.
2. Blotting module (including blotting pads and sponges) and power supply unit.
3. Transfer membrane: Protran BA 85 cellulosenitrate (Schleicher & Schuell).
4. 3MM chromatography paper (Whatman, Fisher).
5. Washing buffer: 18 g of NaCl, 20 mL of 1 *M* Tris (pH 7.6), 10 mL of Tween-20 (10%), to a final volume of 2 L.
6. Membrane-blocking buffer: washing buffer plus 1% BSA.
7. Primary antibodies against the doubly phosphorylated forms of the different

9. Run the gel at 70 V until the samples enter the separating gel, and raise the voltage to 120 V. This procedure should take approx 2–3 h. Stop when the dye reaches the bottom of the gel.

3.4. Blotting

1. Remove the gel to the transfer apparatus. The transfer sandwich is assembled in the following order starting from the cathode (black side): fiber pad, filter paper, the gel, the nitrocellulose membrane, filter paper, and fiber pad (*see* **Note 4**).

2. Transfer for 2 h at 150 mA (constant current) in transfer buffer. The transfer can be carried out overnight, in the cold, at 30 mA. It is recommended to insert a cooling unit in the transfer tank or run it in a cold chamber.

3. Take the blot out, mark its orientation, and rinse in double-distilled water (*see* **Note 5**).

3.5. Blocking and Immunoreaction

1. To reduce the nonspecific binding of the antibodies, incubate the blot in blocking solution for 1 h at room temperature with gentle shaking.

2. Incubate the blots with the primary antibody overnight at 4°C with gentle shaking.

3. Wash three times (5 min each) with washing buffer.

4. Incubate with the secondary antibody for 1 h at room temperature with gentle shaking.

5. Wash three times as in **step 3**.

3.6. Detection

1. Combine the detection solutions 1 and 2 (included in the ECL kit) at a 1:1 ratio to a final volume of 0.1 mL/cm² of the membrane. Mix and add directly to the membrane. Incubate for 1 min at room temperature without shaking.

2. Remove the excess detection solution, wrap the blot with a transparent membrane (e.g., Saran wrap), place it with the protein side up, and smooth out any air bubbles.

3. Expose to an autoradiography film. The first exposure should be a short one (15 s), and then the exposure can be adjusted as needed.

3.7. Stripping and Reprobing of the Membrane

To probe other proteins without running a new gel, it is possible to strip the blot and reprobe it. For instance, it is advisable to probe first with the antibody that recognizes the active, phosphorylated form of the protein, and then strip and reprobe with the antibody that recognizes the general amount of the protein (*see* **Note 6**).

1. Submerge the membrane in stripping buffer and incubate at 50°C for 30 min.

2. Wash the membrane two times (10 min each) in washing buffer.

9. Separate by electrophoresis *(10)* at a constant current of 50 mA until the markers are 0.5 cm from the bottom of the gel.

3.4. Western Blotting and Autoradiography

1. Saturate the 3MM paper (two per gel, approximately the size of the gel to be blotted), blotting pads,, and the transfer membrane in transfer buffer before use (approx 10 min). Take care to press all bubbles from the pads, because bubbles block protein transfer.
2. Create a sandwich with the items in **step 1**, by first placing two sponges in a container filled with transfer buffer to a level slightly above the sponges. Next, place the sheets of 3MM paper between the sponges. Open the sandwich and place the transfer membrane on the 3MM paper, and gently float the gel over the transfer membrane until it is properly aligned with the paper and sponges. Once the gel is in the correct orientation, close the sandwich and place it on the transfer device previously filled with transfer buffer.
3. Transfer for 2 h at a constant 200 mA.
4. Carefully detach the transfer membrane from the gel, and block it with 1% BSA in washing buffer for 1 h at room temperature.
5. React the blot overnight at 4°C or for 2 h at room temperature with the primary antibody against the doubly phosphorylated forms of the signaling cascade under study (ERKs, JNKs, or p38) (*see* **Notes 4** and **5**).
6. Wash the blot with washing buffer three times for 5 min each.
7. Incubate for 1 h at room temperature with the secondary antibody (HRP-linked protein A in the case of polyclonal primary antibodies, or goat antimouse HRP-linked antibody if using monoclonal primary antibodies; dilutions are according to the manufacturer).
8. Wash the blot with washing buffer once for 15 min, and for times for 5 min each.
9. Develop with the ECL kit according to the manufacturer's protocol (*see* **Notes 6** and **7**).
10. To detect the total (phosphorylated and nonphosphorylated forms) amount of the MAPK members, strip the same blots in 0.9% (w/v) NaCl, 10 m*M* Tris-HCl, 0.05% (v/v) Tween-20, and 2% (w/v) SDS (pH 7.6), four times for 10 min each at room temperature under vigorous shaking.
11. Rinse the blots three times for 10 min each in washing buffer (the same stripping buffer without SDS).
12. Block for 1.5 h with 1% BSA in washing buffer.
13. Incubate the blot with the first antibody anti–total ERKs, JNKs, or p38 and follow **steps 2–12**.
14. Perform quantification by using a computerized densitometer and image analyzer (Molecular Dynamics, Sunnyvale, CA) or a regular scanner and the aid of a graphic computer software (such as NIH Image Software).

4. Notes

1. Independently of the experimental model and behavioral protocols used, it is important to dissect and homogenate the brain areas very rapidly (no more than 1 min after decapitation) in order to avoid degradation of the phosphorylation.

2. The Lowry method of protein determination is preferred because of the high amount of β-mercaptoethanol and SDS present in the samples.
3. Do not boil the protein molecular markers unless indicated by the supplier.
4. The antibodies directed against the biphosphorylated forms of the MAPK signaling members are usually applied first (1:1000 dilution is the usual case for commercially available antibodies).
5. The efficacy of the stripping step can be assessed by omitting the first antibody and verifying the lack of signals on the blot.
6. A 1- to 2-min exposure of the blot during the ECL developing should be enough to render a strong signal if using freshly prepared primary and secondary antibodies, and ECL reagents.
7. Developed blots can be stored (sealed in plastic wrap) at 4°C for further use. Dry areas will occasionally develop while attempting later exposures. In this case, the blot should be reincubated with the antibodies and the ECL reagents.

Acknowledgments

References

1. Grewal, S. S., York, R. D., and Stork, P. J. (1999) Extracellular-signal regulated kinase signaling in neurons. *Curr. Opin. Neurobiol.* **9,** 544–553.
2. Sweatt, J. D. (2001) The neuronal MAP kinase cascade: a biochemical signal transduction system subserving synaptic plasticity and memory. *J. Neurochem.* **76,** 1–10.
3. Berman, D. E., Hazvi, S., Neduva, V., and Dudai, Y. (2000) The role of identified neurotransmitter systems in the response of insular cortex to unfamiliar taste: activation of ERK1-2 and formation of a memory trace. *J. Neurosci.* **20,** 7017–7023.
4. Rosenblum, K., Futter, M., Jones, M., Hulme, E. C., and Bliss T. V. P. (2000) ERKI/II regulation by the muscarinic acetylcholine receptors in neurons. *J. Neurosci.* **20,** 977–985.
5. English, J. D. and Sweatt, J. D. (1996) Activation of p42 mitogen-activated protein kinase in hippocampal long term potentiation. *J. Biol. Chem.* **271,** 24,329–24,332.
6. Berman, D. E., Hazvi, S., Rosenblum, K., Seger, R., and Dudai, Y. (1998) Specific and differential activation of mitogen-activated protein kinase cascades by unfamiliar taste in the insular cortex of the behaving rat. *J. Neurosci.* **18,** 10,037–10,044.
7. Atkins, C. M., Selcher, J. C., Petraitis, J. J., Trzaskos, J. M., and Sweatt, J. D. (1998) The MAP kinase cascade is required for mammalian associative learning. *Nature Neurosci.* **1,** 617–609.
8. Blum, S., Moore, A. N., Adams, F., and Dash, P. K. (1999) A mitogen-activated protein kinase cascade in the CA1/CA2 subfield of the dorsal hippocampus is essential for long-term spatial memory. *J. Neurosci.* **19,** 3535–3544.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275.

10. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T$_4$. *Nature* **369,** 156–160.